Lysine based amorphous polyurethanes decorated witn pendant bio-active groups

Yin, Jie

RIJKSUNIVERSITEIT GRONINGEN

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Promotor : Prof. dr. J.A. Loontjens

Beoordelingscommissie : Prof. dr. A.J. Schouten
Prof. dr. D.W. Grijpma
Prof. dr. Ir. W.E. Hennink

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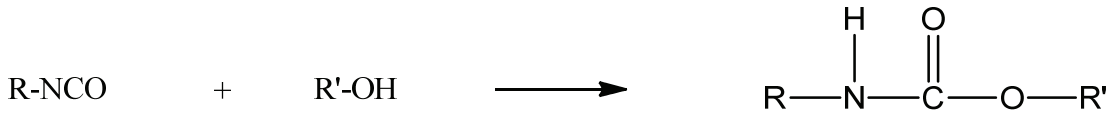
Content

Chapter 1

General introduction

Polyurethanes

Due to a wide range of physical and chemical properties, polyurethanes (PUs) are used in numerous applications like coatings, foams, fibers, engineering plastics and biomaterials.[1-4] The pioneering work on polyurethane polymers was conducted by Otto Bayer and his coworkers in 1937 at the laboratories of I.G. Farben in Leverkusen, Germany.[5] They recognized that using the polyaddition principle to produce polyurethanes from diisocyanates and polyether or polyester diols (polyols, Scheme 1.1) seemed to point to special opportunities, especially when compared to already existing plastics that were made by polymerizing olefins or by polycondensation.



Scheme 1.1 Urethane formation by reaction of an isocyanate with a compound comprising a hydroxyl group

The general used reactants for segmented polyurethanes are a diisocyanate, a polyol and an extender. Segmented polyurethanes can be considered as multiblock copolymers, consisting of a hard and a soft block (segment). The hard segment originates from the diisocyanate and the chain extender, whereas the soft segment is the polyol. The formation of the urethane linkage allows hydrogen bonding between adjacent polymer molecules, where the proton of the carbamate group acts as the hydrogen-bond donor, whereas the oxygen atoms in both the hard- and soft segments can act as hydrogen-bond acceptors, as shown in Figure 1.1.

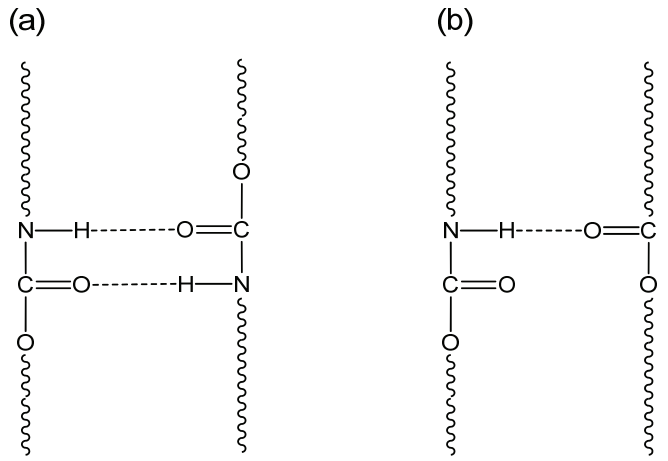


Figure 1.1 Formation of H-bonds in polyurethanes. (a) H-bonds between two urethane groups, (b) H-bond between urethane and ester.

Due to differences in glass-transition temperature, segment polarity, crystalline ability, regularity and chemical composition between the hard and soft segments, a micro phase-segregated morphology will arise as shown in Figure 1.2.[6, 7] Of course the morphology of these systems plays an important role in determining the final product properties. Several factors can influence the morphology of polyurethanes. Factors like crystallization, interphase mixing, and hydrogen bonding in both segments and thermal history, all influence the final morphology of a system. A wide variety of literature have been dedicated to these aspects.[8]

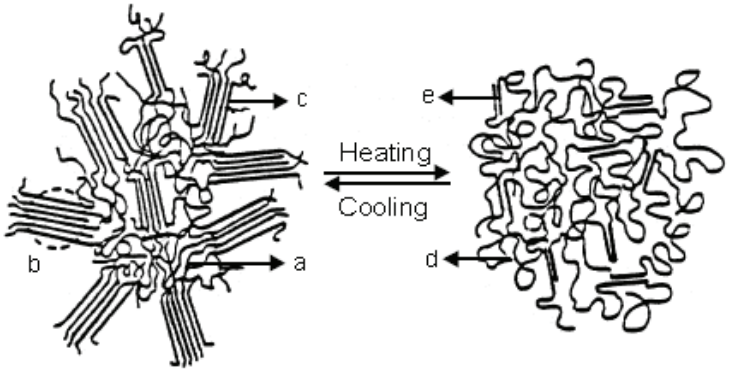


Figure 1.2 Schematic representation of microphase separation of polyurethanes, a: amorphous mixture of hard and soft segments; b: crystalline hard segment domains; c: paracrystalline soft segment; d: disordered soft segment; e: disordered hard segment.[7]

Synthesis of polyurethanes

In industrial- and laboratory-scale syntheses of polyurethanes both bulk and solution polymerisation techniques are used. The bulk polymerisation generally offers higher reaction rates, whereas a polymerisation in solution is more controllable and usually yields higher molecular mass polymers, as the lower viscosity of the reaction mixture overcomes problems caused by diffusion rate control towards the end of the reaction.
On a laboratory scale both methods are used to synthesize polyurethanes via two different routes, as depicted in Figure1.4. In the one-step synthesis (with stoichiometric ratios (1:1) of polymeric diol and diisocyanate) a hard segment with two carbamate groups is formed as shown in the upper part of Figure 1.3.[8] By using the end-cap method with an excess of diisocyanate so-called 3-block-polyurethanes can be synthesized; these 3-blocks consist of chain extender with two diisocyanate molecules. This is essentially a two-step process, in which first a polymeric diol is end-capped with an excess of diisocyanate to avoid chain-extension. Pennings *et al*. used a six-fold excess of the volatile butane diisocyanate (BDI), to ensure that chain extension was suppressed maximally.[9] The excess of BDI was removed by high vacuum distillation. The isocyanate functional oligomers were chain extended with a diol, resulting in the formation of a 3-block-polyurethane with four carbamate groups. The increase in hydrogen bond donor and acceptor groups compared to the one-block polyurethane described previously generally increases the thermal and mechanical properties of the polymer.

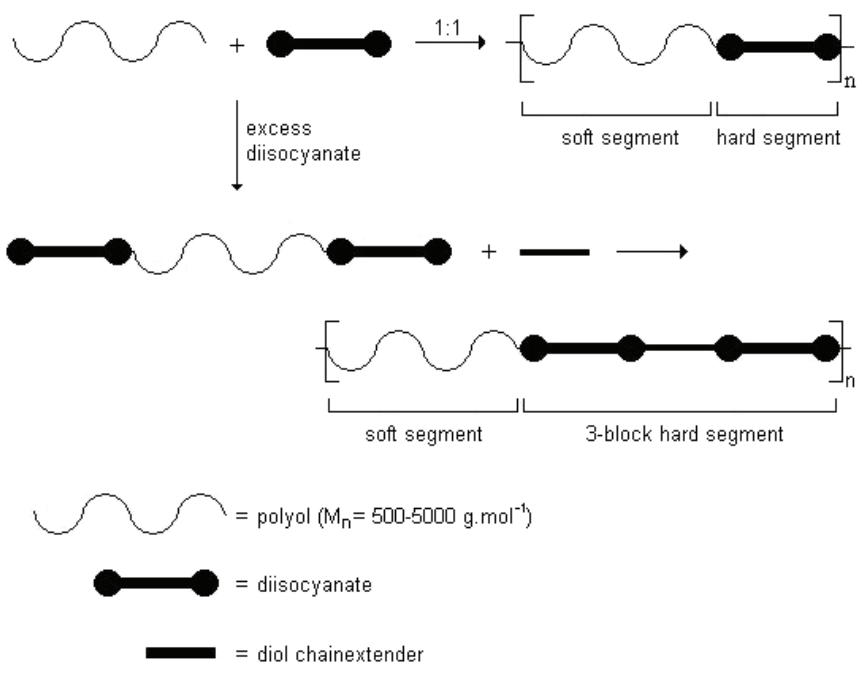


Figure 1.3 Schematic representation of the reaction of a polyester diol with an excess of a diisocyanate and a diol as chain extender[10]

Although polyurethanes prepared by industrial methods are similar in reaction mechanism they will generally have less well-defined hard segments, because it is usually not feasible commercially to use the required six-fold excess of diisocyanate. In the usually employed 'one pot' production process stoichiometric amounts of polymeric diol and chain extender are mixed with a slight excess of diisocyanate. In this process chain extension will readily occur from the start of the reaction and one-, three- and possibly multi-block urethane segments could be formed. Even when the chain extension diol is added after the excess of diisocyanate has reacted with the polymeric diol a certain amount of chain extension may have occurred giving rise to single urethane-blocks. The detrimental effect the use of stoichiometric amounts has on physicochemical properties is usually accepted in favor of a more cost efficient process.[10]

Diisocyanates

Commonly used diisocyanates are diphenylmethane-4-4'-diisocyanate (MDI), toluenediisocyanate (TDI), hexamethylene diisocyanate (HDI) and hydrogenated MDI.[11] One important reason to use these isocyanates is their low volatility. But, more importantly, the polyurethanes based on these diisocyanates have excellent properties.

There is a vast amount of information of the excellent biocompatibility of polyurethanes, making them very suitable for biomedical applications. There are generally two classes in use. For long lasting applications, like for tubes, the polymers should be stable under physiological conditions. In contrast, for tissue engineering the polymers should be biodegradable in a controlled way. Depending on the composition, polyurethanes can fulfill all these requirements, but have to be selected accordingly.[12]

The on large scale commercial available diisocyanates (MDI, TDI, HDI, H-MDI) are also used for biomedical polyurethanes even though it is known that the corresponding polyurethanes release upon degradation toxic and carcinogenic diamines, particularly the aromatic diamines, originating from the aromatic diisocyanate.[13] Especially for biomedical applications, new aliphatic diisocyanate were developed, for instance 1,4-butanediisocyanate (BDI) and lysine diisocyanate (LDI). Polyurethanes based on BDI and LDI are a good non-toxic alternative since, upon degradation, the formed diamines are naturally occurring substances in the body and therefore considered safe to use *in vivo*. Butane diamine and its homologues (spermidine and spermine) are non-toxic polyamines that are essential for cell growth and differentiation.[14]

Polyurethanes based on LDI

Lysine diisocyanate (LDI) (Figure 1.4) is commercially available only from Kyowa Hakko Kogyo Co., (Tokyo, Japan). Numerous studies have been reported on the synthesis and properties of a range of polyurethanes based on lysine diisocyanate (LDI).[15-50] Those materials are biodegradable and non-toxic, which can be used in the plenty of biomedical applications, especially in tissue engineering.[25]

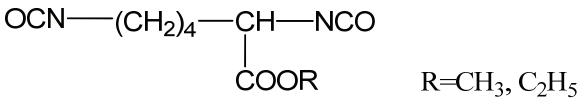


Figure 1.4 Structure of lysine diisocyanate

Hirt *et al*.[26] and de Groot *et al*.[27] reported on the synthesis and properties of degradable polyurethanes based on LDI, 2,2,4-trimethyl-hexamethylene diisocyanate and a number of polyester and copolyester polyols such as Diorez®, caprolactone-ethylene glycol copolymers and poly hydroxy butyrate and valerate copolymers. The polyurethanes ranged from elastomers with elongations at break as high as 780 %, but with low tensile strengths (5.8 to 8.1 MPa). Saad *et al.*[16] reported on the cell and tissue interaction of four such polymers prepared from 2,2,4-trimethylhexamethylene diisocyanate and LDI, and polyols like ω-dihydroxy-poly(R-3-hydroxybutyrate-co-(R)-3-hydroxyvalerate)-block-ethylene glycol] and two commercial diols, Diorez® and PCL-diol. *In vitro* studies indicated that these polyesterurethanes did not activate macrophages and showed good level of cell adhesion and growth, which were also confirmed by *in vivo* results. Structure-property relationships of degradable polyurethanes based on LDI, polycaprolactone, polyethylene oxide (PEO) and an chain extender have been investigated by Skarja and Woodhouse.[17, 49] Their results showed that the strength of PEO based polyetherurethane (PEU) were generally weaker but PCL based materials were relatively strong. However, no results were reported on the degradation of these polyurethanes

Storey *et al.*[20, 30] have prepared poly(ester urethane) networks from LDI and a series of polyester triols based on dl-lactide, γ-caprolactone and their copolymers. Networks based on poly(dl-lactide) were rigid (glass transition temperature Tg = 60ºC) with ultimate tensile strengths of 40 to 70 MPa, whereas those based on caprolactone triols were low modulus elastomers with tensile strengths of 1 to 4 MPa. Networks based on copolymers were more elastomeric (elongation up to 600%) with compressive strengths between 3 to 25 MPa. Hydrolytic degradation under simulated physiological conditions was dependent on the type of triol. Dl-Lactide based networks were the most resistant. No degradation was observed after 60 days. Caprolactone based triol networks were resistant up to 40

days. The high lactide containing copolymer networks were most susceptible for hydrolysis and substantial degradation was observed in about 3 days.

Bruin *et al.*[50] have reported on the synthesis of degradable polyurethane networks based on star-shaped polyester prepolymers. The star-prepolymers were prepared from myoinisitol, a pentahydroxy sugar, as initiator molecule for the ring-opening copolymerisation of l-lactide or glycolide with caprolactone. The prepolymers were cross-linked using LDI. The degradation products of these PU networks were considered to be non-toxic. The resulting network polymers were elastomeric with elongation in the range 300 to 500% and tensile strengths varying between 8 to 40 MPa depending on the branch length. Preliminary experiments in guinea pigs have shown that the polyurethanes biodegrade when implanted subcutaneously. Polyurethane networks based on LDI and poly(glycolide-co-γ-caprolactone) macrodiol were evaluated by Bruin *et al.*[28] as two-layer artificial skin. The degradation of the skin *in vivo* was faster than that *in vitro*. Subcutaneous implantation in guinea pigs showed that the porous polyurethane networks allowed rapid cell in-growth, degraded almost completely 4-8 weeks after implantation and evoked no adverse tissue reaction. Zang *et al.*[29] have developed a peptide based polyurethane scaffold for tissue engineering. LDI reacted first with glycerol to form a prepolymer, which upon reaction with water produced a cross-linked porous sponge, due to liberation of carbon dioxide. Initial cell growth studies with rabbit bone marrow stromal cells have shown that the polymer matrix supported cell growth and was phenotypically similar to those grown on tissue culture polystyrene.

Many diols or polyols (or copolymers thereof) have been used to synthesize polyurethanes based on LDI. The most commonly used polydiols are showed in Figure 1.5.

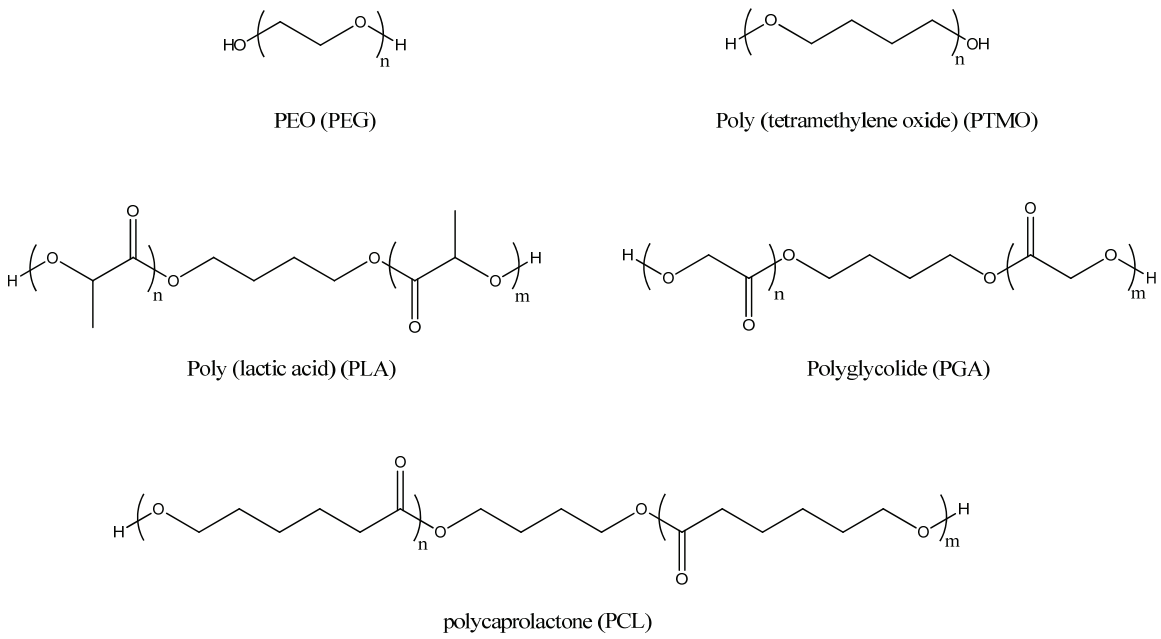
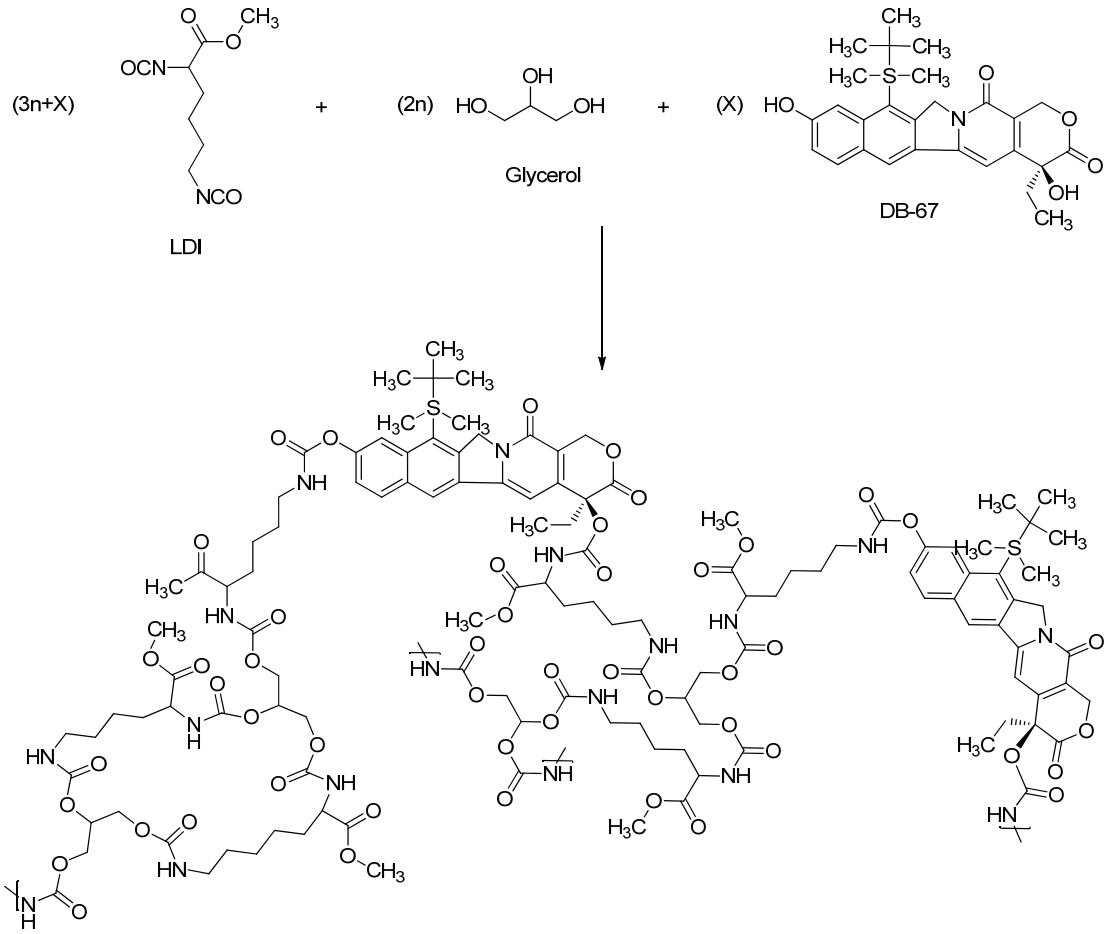


Figure 1.5 The chemical structure of most commonly used hydroxyl functional compounds (and copolymers thereof) for synthesizing polyurethanes based on LDI.

Recently, biodegradable LDI based polyurethanes for drug delivery have been developed.[36-50] Beckman's group prepared a new generation of biodegradable polyurethane discs and foams based on LDI and glycerol and demonstrated their application as long-term drug delivery reservoirs.[36-40] They have previously showed that polyurethanes synthesized from LDI and glycerol degrade hydrolytically into the non-toxic components lysine, glycerol and CO_2.[29] The drugs 7-tert-butyldimethylsilyl-10-hydroxy-camptothecin (DB-67) or Doxorubicin were incorporated into the LDI-glycerol polymer network via hydrolysable urethane linkages. For example, Scheme 1.2 illustrates the chemical structure of DB-67 contained component and a proposed polymer structure.[36]



Scheme 1.2 Molecular structure of a polyurethane polymer network from DB-67, LDI and glycerol.[36]

During the hydrolysis of polyurethanes, the drugs will be released. Several parameters can influence the hydrolysis rate of polymers like molar ratio of monomers, temperature and so on. By varying the composition and thereby the hydrolysis rate of polyurethanes, the drug release rate can be tuned. However, these systems are essentially based on the formulations of polyurethane matrices, of which applications are limited due to their burst release effect and localized drug delivery.

Ding *et al*.[40-44] have developed a new group of LDI based cationic biodegradable multiblock poly(3-caprolactone urethane)s bearing gemini pendant quaternary ammonium groups and methoxy-poly(ethylene glycol) (m-PEG) end chains (Figure 1.6).

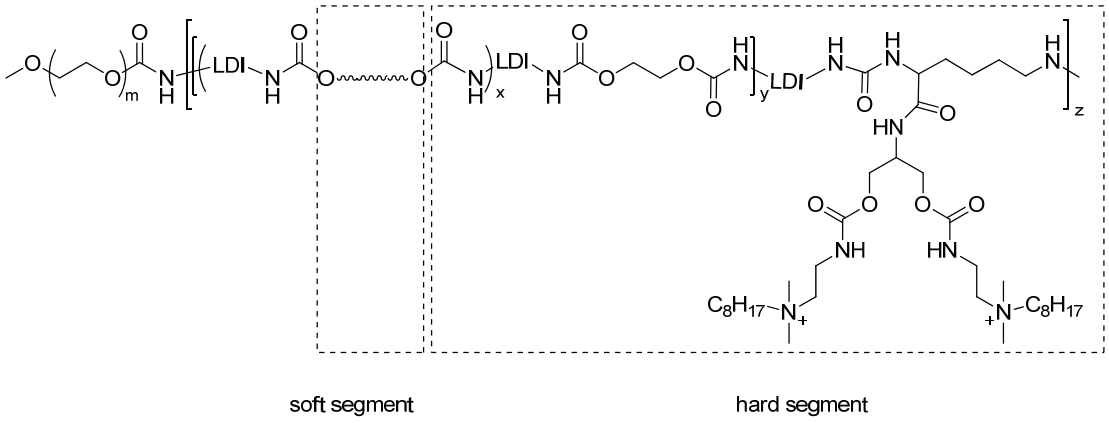


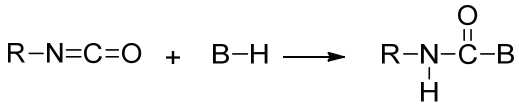
Figure 1.6 Schematic structure of multiblock of poly(ε-caprolactone), LDI and cationic functional lysine. ∿∿∿ represents the poly(ε-caprolactone) soft segment.[44]

The incorporation of gemini quaternary ammonium groups has significant impacts on the properties of multiblock poly(ε-caprolactone urethane)s, such as a decrease of the PCL segment crystallinity, enhancement of surface hydrophilicity, and water sorption. Those gemini polyurethanes were able to self-assemble into a micellar structure with a diameter below 100 nm, at a low critical micelle concentration (CMC) in aqueous solution. These nanocarriers with highly tunable micellization characteristics, high drug loading capacity, efficient cellular uptake and good biocompatibility were proposed as excellent biodegradable candidates in drug and gene delivery as well as for imaging applications.

Polyurethanes based on blocked diisocyanate

It is convenient to synthesize polyurethanes from diisocyanates (DIs) since the DIs are reactive with alcohols at low temperature, when catalyzed, and with amines without catalysts. However, there are several major drawbacks. The high reactivity and toxicity of isocyanates do not allow for their storage and use in one-pack systems, which are not desirable in industry and in biomedical applications.[51, 52] More importantly, due to the high reactivity, it is not possible to have or introduce moieties that active hydrogens, like hydroxyl groups, amine of acids, while keeping the isocyanate groups intact. Blocked isocyanates provide a solution for overcoming these drawbacks. The isocyanate

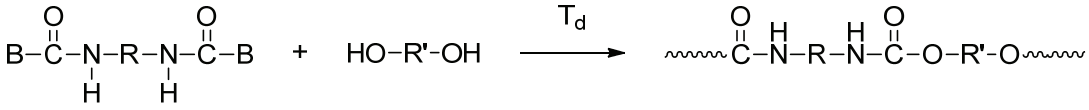
functional group can be blocked or protected by a number of methods. Commonly, blocked isocyanates are prepared by reacting them with an active hydrogen compound Scheme 1.3).[52]



Scheme 1.3 *A blocked isocyanate by reaction with active hydrogen compounds B-H (blocking agent).*

Upon heating in the presence of a nucleophile (-OH or $–NH_2$), the blocking group is released and the desired urethane/urea is obtained (Scheme 1.4). Important review papers from Wicks *et al*.[51] have extensively described the chemistry of blocked isocyanates and discussed the mechanisms of the different blocking methods in detail. Following them, the uses and applications of blocked isocyanates in coatings and non-coatings fields are reviewed.[52, 53] Within the market, blocked isocyanates have wide spread use in many coatings areas: automotive, maintenance, and industrial finishing.

The regenerated isocyanate can react with a coreactant containing hydroxyl functional groups to form urethanes, which are thermally stable bonds. A typical scheme for making PUs from blocked isocyanates is depicted in Scheme 1.4.

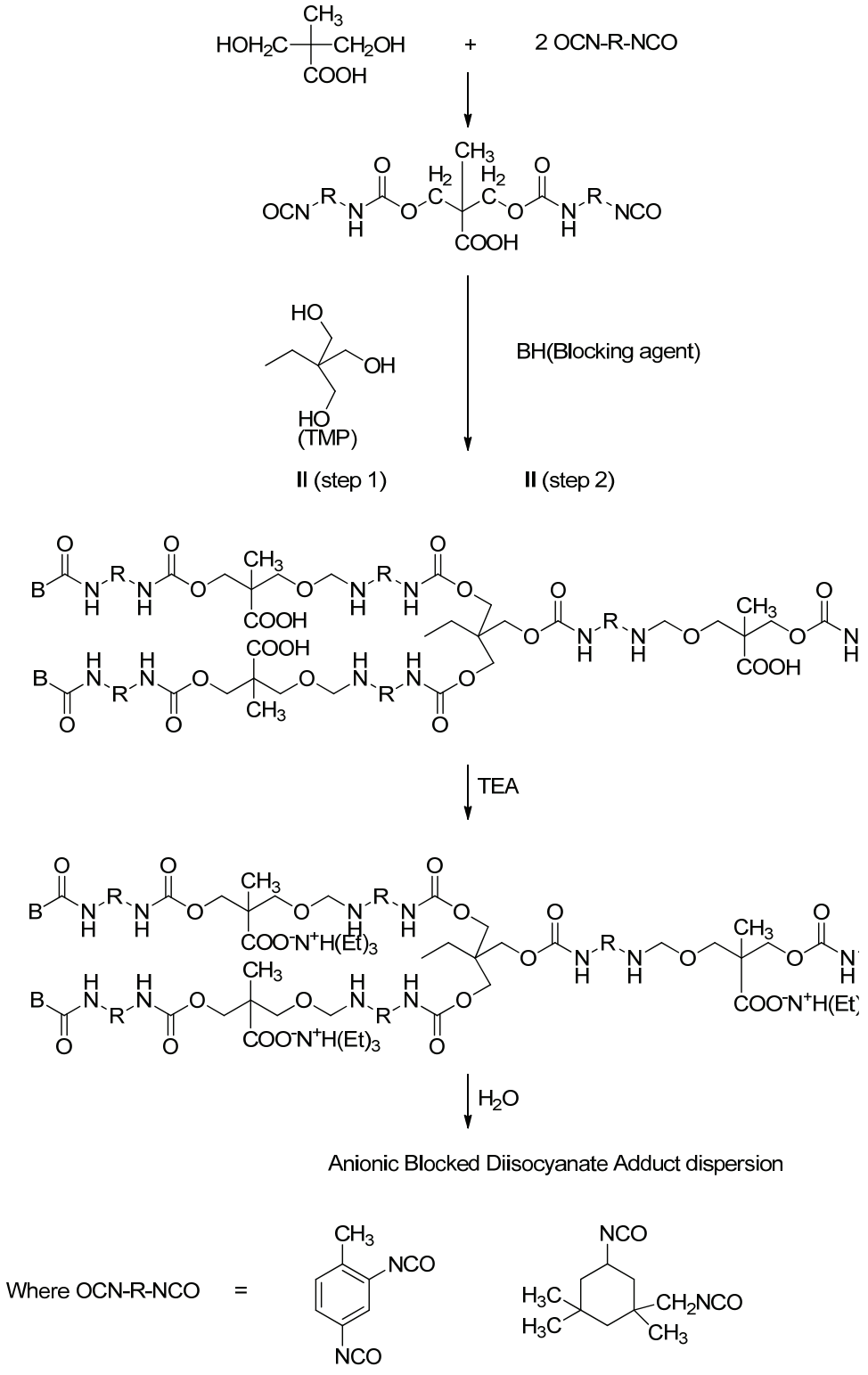


Scheme 1.4 Formation of linear polyurethane based on a blocked diisocyanate and diol, where T_d is the deblocking temperature.

Generally, commercial blocking agents include phenol, ε-caprolactam (CL), methyl ethyl ketoxime (MEKO), dimethyl pyrazoles, diethyl malonates, sodium bisulfite, and many others.[52] The blocking group determines mainly the deblocking temperature, which is generally between 50 and 160 ^{o}C. The type of nucleophile has an influence on the blocking temperature as well, but less dominant than the blocking group. Polyurethanes

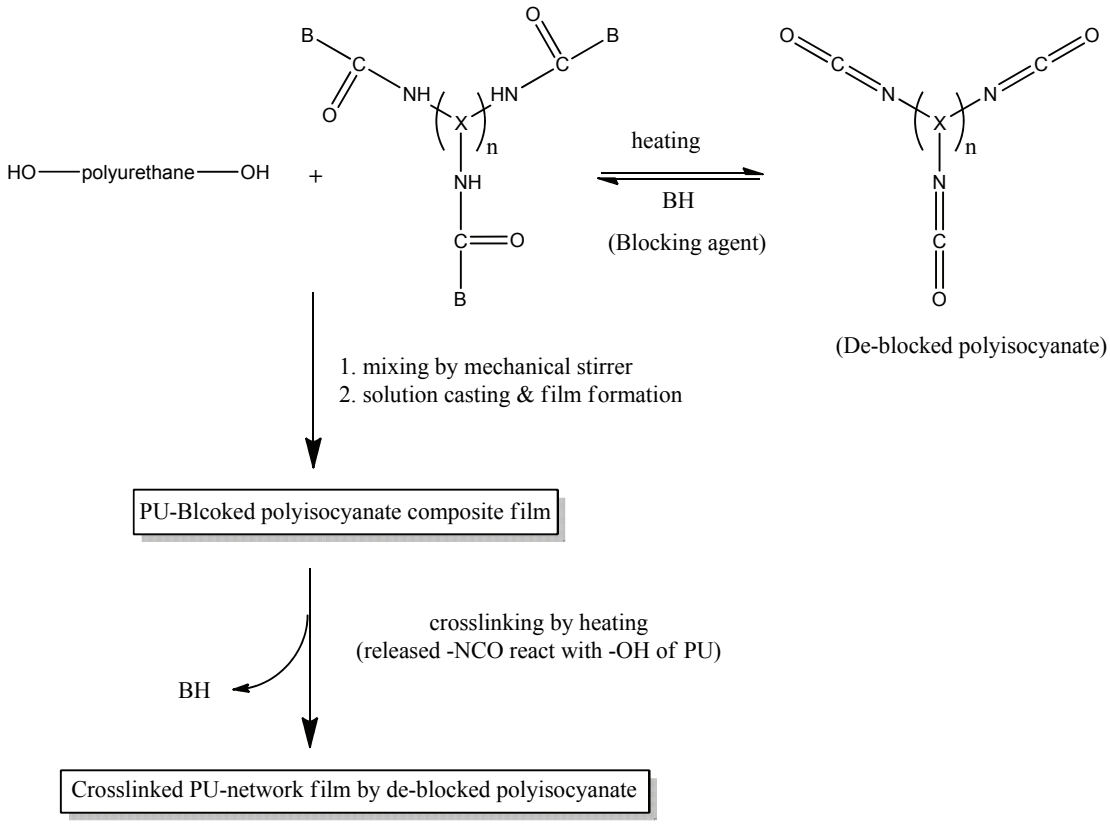
based on blocked isocyanates have been described in numerous applications, like waterborne, coatings, adhesives, sealants, textile, paper, plastics and so on.

The use of blocked isocyanates in coatings is exemplified by the work of Shen *et al*.[54] who reported a series of water-dispersible blocked polyisocyanates that were synthesized from toluene 2,4-diisocyanate (TDI), isophorone diisocyanate (IPDI), dimethylol propionic acid, methyl ethyl ketoxime (MEKO), ethyl cellosolve (EC), and ε-caprolactam (CL) (Scheme 1.5).



Scheme 1.5. Synthesis of the anionic blocked-isocyanate adducts.[54]

All aqueous dispersions of the blocked polyisocyanates showed good storage stability. In this study, both the blocked TDI- and IPDI-based polyisocyanates started to deblock at about 55–85^{o}C. The deblocking temperature increased, in the order: MEKO < EC < CL. These blocked-isocyanate adducts deblocked and were crosslinkers of hydroxyl–polyurethane emulsions (HPUE) at different deblocking temperatures. The excellent water resistance and high tensile strength of the composite films confirmed that HPUE films were well crosslinked (Scheme 1.6).



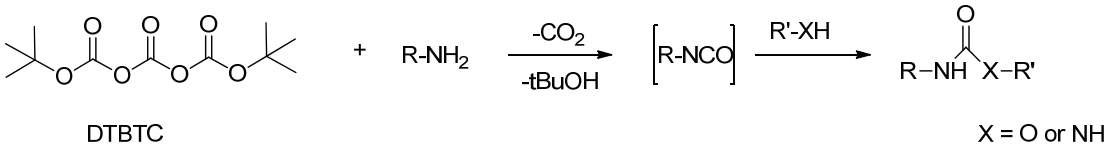
Scheme 1.6. Function of the blocked polyisocyanate as crosslinker in the PU film.[54]

Polyurethane based on Isocyanate-free method

So far most of blocked isocyanates are synthesized from isocyanates. To avoid this detour and the use of these toxic DIs and to enable the introduction of functional groups

researchers have been exploring different ways to prepare polyurethanes via isocyanate-free strategies. Several routes have been developed to prepare polymers, for example by using di-tert-butyltricarbonate (DTBTC), cyclic carbonates, dicarbamates and carbonylbiscaprolactam (CBC).

DTBTC is a reactive compound which can be used to prepare polyurethane/urea, as has been described by Versteenen *et al*.[55-57] DTBTC is a well-known compound used as blocking groups of the amines in amino acids. The amino group reacts with DTBTC to form an isocyanate, even at room temperature (Scheme 1.7).

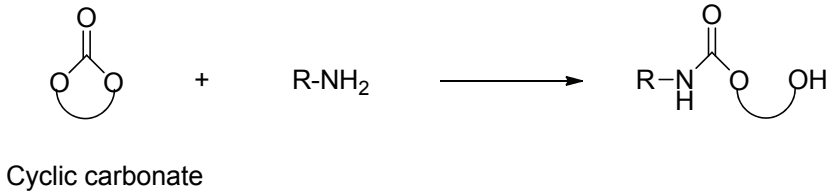


Scheme 1.7 Chemistry of DTBTC to prepare polyurethanes or polyureas.[57]

If diamine is used in a stoichiometric amount with DTBTC, polyureas are obtained. When an amino alcohol is employed to react with DTBTC, an intermediate isocyanate alcohol forms, followed by polymerization reaction between the hydroxyl group and the isocyanate group, when catalyzed by a lewis acid. The authors also reported that by using this technology, segmented poly(ether urea)s with uniform hard segments can be obtained. This method is obviously also based on isocyanate chemistry but the toxic compounds are formed *in situ*, thus it can be performed without handling DIs as starting materials. However, the residual free isocyanate groups cannot be avoided.

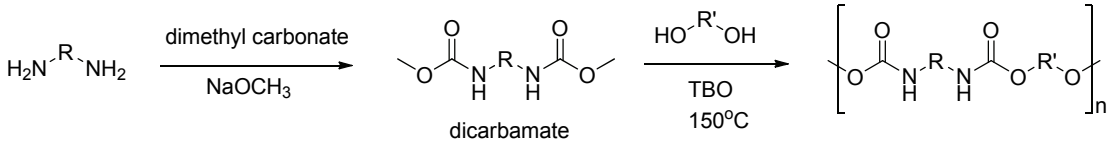
Another isocyanate-free route to prepare PUs is using cyclic carbonates as starting materials. The low toxicity, the biodegradability of cyclic carbonates and their high reactivity with amines make them very attractive to researchers in the field of isocyanate-free PUs, as the review paper from Guan et al.[58] showed. In this cyclic carbonate strategy, using only diamines and cyclic carbonates, it is unavoidable that carbonate groups are also obtained in the PU due to the presence of hydroxyl groups originating from the reaction between cyclic carbonates and amines (Scheme 1.8). This method can result in

lower crystallinity because the carbonate groups could hinder the crystallization of hard segments.



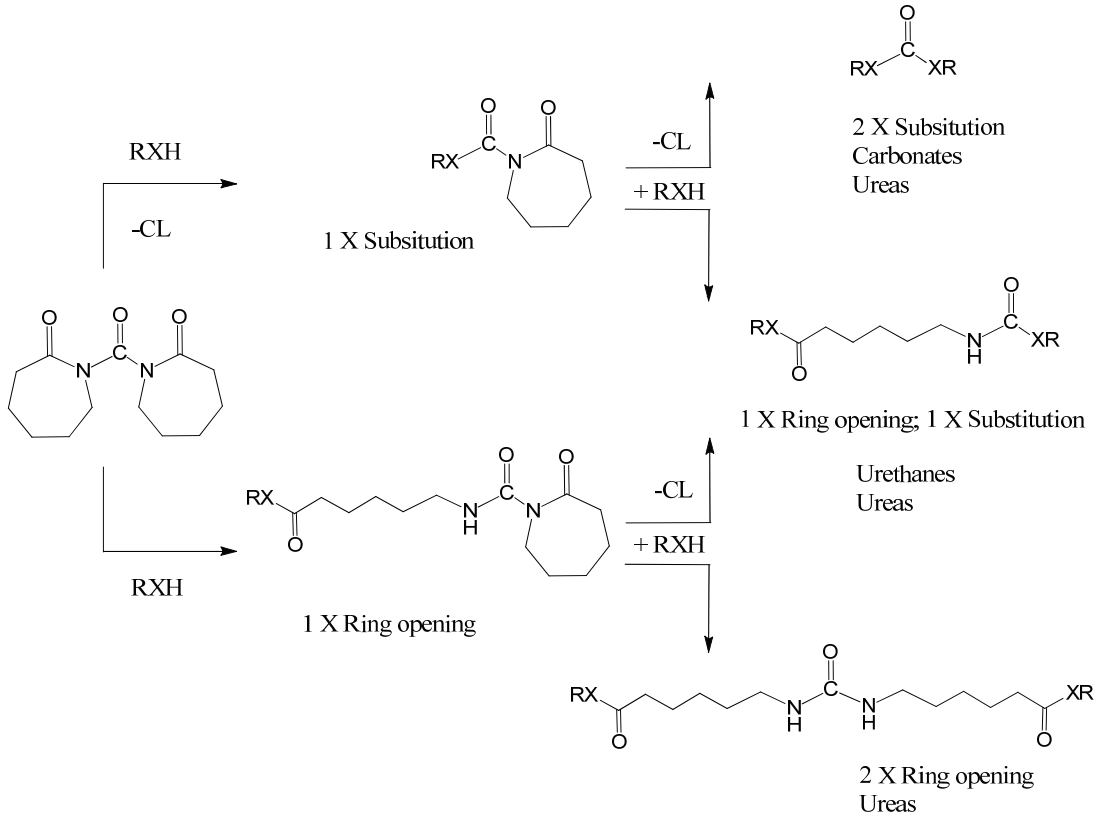
Scheme 1.8 Reaction between cyclic carbonates and amines.

Dicarbamates can also be employed as the starting materials for the synthesis of PUs, as non-toxic substitutes for DIs. As can be concluded when comparing the chemical structures of dicarbamates (Scheme 1.9) and diisocyanates, dicarbamates can be obtained from diamines by reacting them with dicarbonates, such as dimethylcarbonate. Deepa et al.[59] reported that PUs can be prepared by polycondensation of dicarbamates with diols or diamines, catalyzed by titanium tetrabutoxide (TBO).



Scheme 1.9 Polycondensation process for synthesis of polyurethanes from dicarbamates in ref.[59]

Loontjens and coworkers[60-63] developed an isocyanate-free route which is based on carbonyl biscaprolactam (CBC, Scheme 1.10). In this route, when CBC reacts once with an alcohol or an amine, caprolactam blocked isocyanates were formed either by ring-opening or ring-elimination reactions. The caprolactam (CL) ring-opening and the CL ring-elimination reactions of CBC can be controlled by the type of nucleophile. Furthermore, upon heating, the CBC-end capped amine (blocked isocyanate) can eliminate another molecule of CL to form an isocyanate group that resulted in polyurethanes or polyurea, which looks promising for powder coating applications.[64]



Scheme 1.10 The possible reactions between carbonyl biscaprolactam and hydroxyl (X = O) or amino (X = NH) functional compounds (CL = ε-caprolactam).

Using the CL blocked isocyanates allowed to make one–package formulation, with the required properties, since there were no volatile (aliphatic) isocyanates present. Most diamines can be modified by using this method. The amino acid lysine for example was chosen in this thesis.

Biomedical polyurethanes

PUs have been extensively investigated as materials for biomedical devices.[65] They were first used in biomedical applications in the late 1950s to construct a composite PU foam breast prosthesis. By the early 1960s, catheters and pacemaker lead insulators were being made out of PUs. The popularity of PUs for use in medical applications has risen steadily ever since. Polyurethanes are generally considered to have an excellent biocompatibility,

and PU chemistry is so varied that it enables fine-tuning of the chemical and physical properties for any given application. Polyurethane copolymers or segmented PUs represent an important subclass of thermoplastic polymers that is frequently used in biomedical devices. These materials tend to be elastomeric, making them particularly well suited for soft tissue engineering applications, where a matrix is needed that can withstand local mechanical forces. The construction of a cardiac patch is one such an example.[65]

Avocathane®, Biomer®, and Tecoflex® are all poly(ether urethanes) based on the poly(tetramethylene oxide) (PTMO).[66] They appeared biocompatible and degraded *in vivo* by biologically assisted environmental stress cracking and metal ion-supported oxidation leading to calcification.[67] When the extracts during degradation of Pellethane® were analyzed, 4,4'-methylene dianiline (MDA) was detected. MDA is known to be carcinogen, mutagen and displays immediate cell toxicity. As mentioned before, upon degradation polyurethanes are able to release toxic and carcinogenic aromatic diamines, originating from the aromatic diisocyanates. The aliphatic diisocyanate for instance, 1,4-butanediisocyanate (BDI) and lysine diisocyanate (LDI) obtained from natural occurring diamines, are therefore beneficial and were developed for biomedical polyurethanes.

Research aims and scope

The aim of this research was to synthesize linear or crosslinked polyurethanes from caprolactam blocked lysine diisocyanate (BLDI), which were provided with functional side groups. A novel synthetic methodology to prepare BLDI will be developed. By making use of the expected stability of BLDI and the presence of a pendant ester group, the introduction bio-functional side groups via that pendant ester group will be studied. The synthesis of amorphous polymer networks will be studied to avoid remnant polymer crystals after degradation. Some preliminary experiments with human cells will be employed to demonstrate the biocompatibility of the resulting polyurethanes.

Chapter 1 give the general introduction to this thesis.

In **chapter 2** an efficient route for the synthesis of caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe), and the modifications and a detailed characterization of BLDI-OMe is described.

In **chapter 3** the polymerization behavior of the caprolactam blocked lysine diisocyanate methyl ester with various diols is reported.

Chapter 4 deals with the synthesis of transparent polyurethane networks, which were prepared by using a one shot technique. The novel blocked lysine diisocyanates (synthesized as described in chapter 2) were used instead of toxic diisocyanates. The polymer networks were fully characterized.

Chapter 5 describes the synthesis of poly (ester-urethanes), comprising activated N-hydroxysuccinimide (NHS) pendant esters, which were prepared from the NHS-activated ester of caprolactam blocked lysine diisocyanate and polycaprolactone. The NHS-activated ester offered an enabled technology to prepare bio-active polyurethanes. Hexyl amine, as model compounds, and a cell-adhesion promoting peptide, GRGDG, were used to substitute the NHS-group, demonstrating the ability of the concept. Human cells adhered well on these GRGDG functional surfaces.

Chapter 6 the synthesis of biotinylated blocked lysine diisocyanates with short alkyl and long polyoxazolines spacer and corresponding polyurethanes is reported. Making use of the strong avidin-biotin binding opportunity, these materials can be easily diversified for a variety of different applications.

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Chapter 2

Synthesis of Caprolactam Blocked Lysine Diisocyanate Methyl ester and its Derivatives

Abstract

An efficient route for the synthesis of caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe), derivatization and a detailed characterization thereof was successfully accomplished. The synthesis of BLDI-OMe was based on the highly selective reaction between compounds comprising primary amines and carbonyl biscaprolactam (CBC). In a highly selective reaction step only one of the two caprolactam rings of CBC was substituted by the amino groups of lysine, yielding the methyl ester of caprolactam blocked lysine diisocyanate. The structure and the purity of BLDI-OMe were determined by ^{1}H-NMR, ^{13}C-NMR, elemental analysis and HPLC. The accordingly determined purity was higher than 99%. A successful model reaction showed that octanol substituted selectively only the caprolactam blocking groups, while leaving the methyl ester unaffected, demonstrating the possibility to make linear polyurethanes with pendant ester groups. It was essential to omit the commonly used tin catalyst, as the ester reacted with octanol in the presence of this catalyst. Crucial for the aim of this work was that conditions were found to hydrolyze the ester group of BLDI-OMe without affecting the blocked isocyanate groups. So, depending on the reaction conditions either the blocked isocyanate group or the ester was reactive. The free acid group could be converted into the N-hydroxysuccinimide (NHS) activated ester. The activated ester allowed further modification with various nucleophiles, as was demonstrated by the substitution of the NHS-group with hexyl amine, as a model reaction, without affecting the blocked isocyanate groups.

2.1. Introduction

Due to the aging of the world population and because it is not accepted anymore to live with loss of function due to trauma or wear, the use of biomedical implants is rapidly growing. Biomedical implants can be divided in short and long lasting applications. For instance in hip replacements, the applied polymers should be very stable, resisting degradation. In contrast, in tissue engineering only temporarily supports are needed. During the recovery of the tissue the polymeric support must degrade in a controlled way. In addition, the polymers have to be biocompatible and must fulfill biological requirements. Highly biocompatible implants prevent active immune responses. During degradation the body will be exposed to the degradation products, which should be non-toxic as well. The polymers must therefore be composed from compounds that do not afford toxic degradation products.

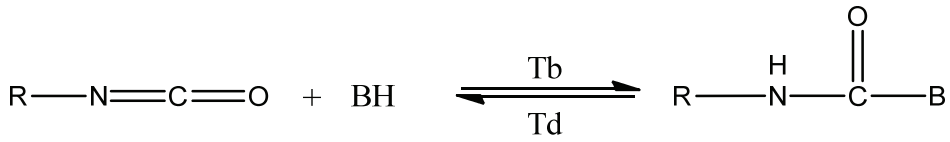
Polyurethanes (PUs) are prepared from diisocyanates and polyols. They have been shown to be very suitable for biomedical implants[1]. Moreover, the properties of PU can be designed widely, fulfilling the demanding requirements of biomedical materials. However, so far the introduction covalently fixed bio-active compounds in polyurethanes has shown to be difficult. Due to the high reactivity of isocyanates the presence of bio-active groups or the introduction thereof is hampered seriously. Blocking the isocyanate groups could be an attractive alternative, while still affording the same polyurethanes with polyols. They are less reactive and therefore possibly suitable to introduce bio-active moieties.

2.1.1. Blocked diisocyanates

Isocyanates are highly toxic due to their ability to react with proteins[2], deactivate enzymes[3-5], destroy cells[6] and damage DNA[7]. Most aliphatic isocyanates are volatile compounds that can invade into the body by inhalation[8]. Diisocyanates are the most frequently reported cause of chemically induced occupational asthma[9] and deterioration of lung function after long-term repeated exposure[10].

The high reactivity and high toxicity of isocyanates neither allow their storage or use in one-component systems nor tolerate the introduction of functional groups[11]. A solution to overcome these problems, particularly used in the coating and paint industries, is the use of blocked isocyanates.[12] Blocked diisocyanates react with polydiols in exactly the same way as unprotected diisocyanates, affording the same polyurethanes, but only with a lower rate. Blocked isocyanates are very a versatile class of materials with many industrial applications. As many as 90% of the references on blocked isocyanates found by Scifinder are patents, which shows their industrial importance.[13,14] Blocked isocyanates have a bright future in the field of powder coatings and heat setting adhesives.[15,16] Both aliphatic and aromatic isocyanates can be blocked by variety of blocked agents.[13,17,18] The most widely commercially used agents are phenols, oximes, ε-caprolactam and dibutyl malonate.[13,19] The conventional route to prepare blocked isocyanates is by the reaction shown in Scheme 1. This procedure depends on the (limited) availability of the desired isocyanates. Moreover, due to the high reactivity of isocyanates the presence of active hydrogen moieties (-NH-, -OH or -COOH) is not feasible. Hence, the preparation of functional isocyanates via this route is hampered seriously.

In contrast, blocked isocyanates might allow chemical modifications, without destroying the isocyanate function. However, the same limitation arises when the blocked isocyanates are prepared from isocyanates (Scheme 2.1).



Scheme 2.1 Reaction of an isocyanate with a blocking agent (BH) to form a blocked isocyanate :Tb = blocking temperature; Td = de-blocking temperature

To circumvent these severe limitations, we studied an alternative methodology in which blocked isocyanates were prepared by the reaction of primary amino groups with carbonyl biscaprolactam (CBC), circumventing the use of free isocyanates.

2.1.2. Blocked diisocyanates from amines and CBC

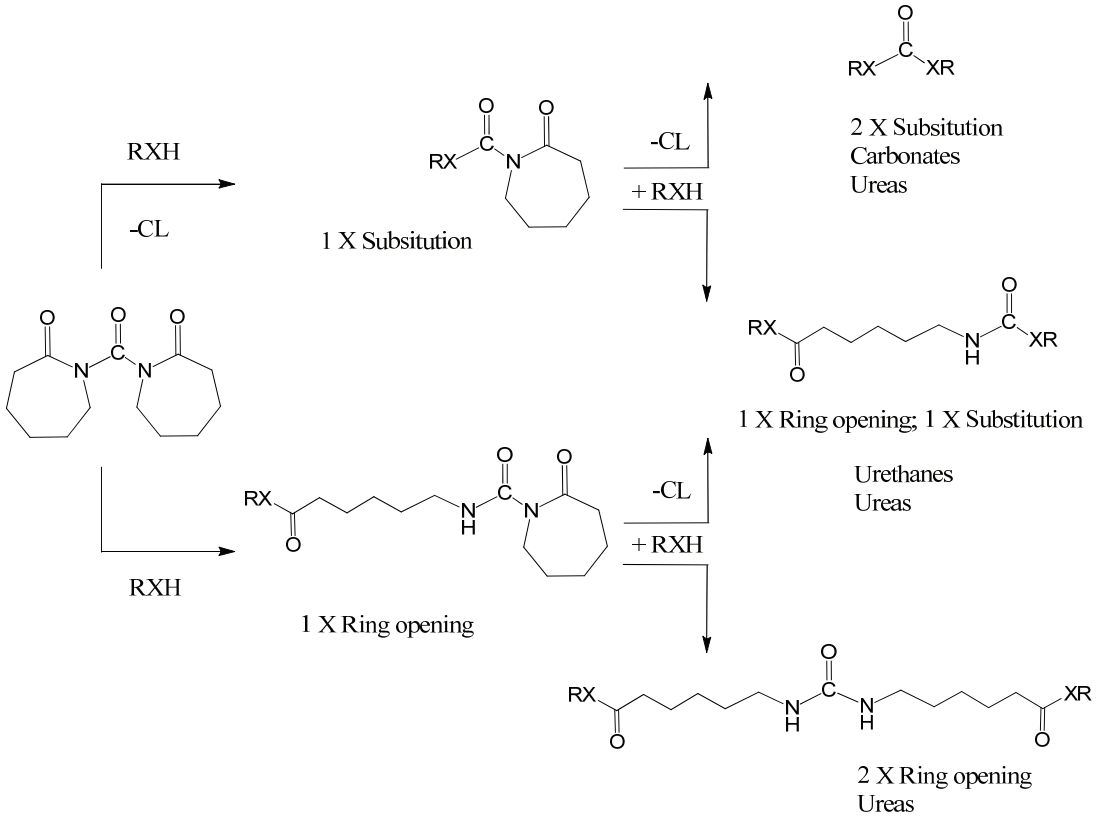
Carbonyl biscaprolactam (CBC) is a nontoxic solid derivative of carbonic acid, which was for the first time prepared by Meyer in 1956[20] and later commercialized by DSM under the brand name Allinco®[21]. It is composed of two caprolactam rings connected onto carbonic acid, and is described as a chain extender to increase the molecular weight of polyamides during reactive processing in an extruder. Early attempts by Meyer to prepare high molecular polymers from CBC and diamines failed and only ill-defined oligomeric products were obtained. During the 1990s, Mateva *et al*. and researchers at Monsanto used CBC as an activator, together with sodium caprolactamate, in the anionic ring opening polymerization of caprolactam for preparing high molecular weight polyamides.[22,23] Müller et al. used CBC as a special bleaching agent.[24,25] Loontjens *et al*., revised the potential of CBC and found that, besides being an excellent chain extender for polycondensates, it was a versatile reagent for isocyanate- free urethane chemistry. [21,26]
CBC offered a new general methodology to prepare (functional) blocked isocyanates (BIs). Two novel routes were discovered to prepare caprolactam-blocked isocyanates. One was based on the reaction of amino groups and CBC, whereas the other was from the reaction of hydroxyl groups and CBC.

2.1.3. Chemistry of CBC

N-acyl lactams are activated (di) acids derivatives, reacting readily with hydroxyl and amino functional compounds, yielding esters and amides respectively. The simplest member of this class is carbonyl biscaprolactam (CBC), in which two caprolactam rings are linked onto carbonic acid. CBC belongs to the class of caprolactam activated diacids, which yield normally ester and amides in the reaction with hydroxyl or amino functional compounds. However in contrast, the reaction of amino and hydroxyl groups with CBC yields blocked isocyanates. This opens an alternative route towards blocked isocyanates that are not obtainable by using isocyanates.

Reactions of the caprolactam ring

According to Loontjens *et al.* caprolactam-blocked isocyanates (CBIs) can be obtained from reactions of amines and alcohols with CBC.[26] If the reaction goes to completion four different consecutive reactions can occur: 1. ring substitution-ring substitution, 2. ring substitution-ring opening, 3. ring opening-ring substitution, 4. ring opening-ring opening (Scheme 2.2).

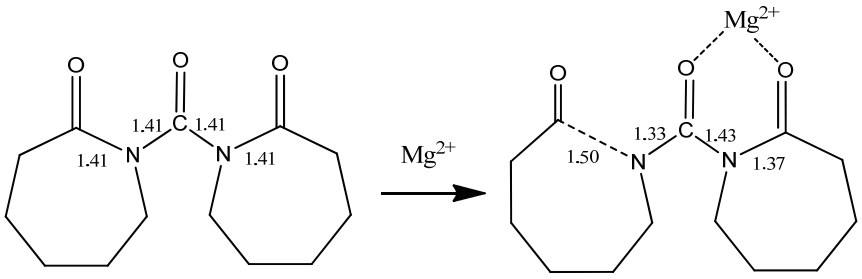


Scheme 2.2 The possible reactions between carbonyl biscaprolactam and hydroxyl (X = O) or amino (X = NH) functional end groups (CL = ε-caprolactam).[20]

Some of the possible intermediate products, after the first reaction steps, are blocked isocyanates. Interestingly, it was found that, at temperatures below 100 ^{o}C, primary amines substitute quantitatively only one of the caprolactam rings of CBC, yielding blocked isocyanates.

When using alcohols, the first caprolactam ring of CBC opens, yielding blocked isocyanates as well. In that case the nitrogen atom of isocyanate group originates from

caprolactam. Due to the less activity of hydroxyl groups compared to the amino groups, a higher temperature (125 ^{o}C) is needed. The reaction is not only less reactive, but also less selective than with primary amines. To have reasonable yields, catalysts (example Mg^{2+}) are necessary. Molecular modeling has shown that the coordination of two of the three carbonyl groups of CBC with metal ions is energetically favorable. The central carbonyl group and one of the ring carbonyl groups operate as a bi-dental ligand, forming a complex with the metal ion. According to the calculations the carbon-nitrogen bond length in the second, non-complexed caprolactam ring is enlarged from 1.41Å to 1.50 Å, suggesting that this bond is weakened. Due to this weakening it is reasonable to expect that this caprolactam ring is more inclined to open in the presence of a catalyst. So far it was found that the second caprolactam ring will always be substituted by hydroxyl of amino groups, which is the common reaction with blocked isocyanates.[20, 26]



Scheme 2.3 The calculated atom-atom distances (in Å) in the complex of CBC with Mg^{2+} ions.[20]

Thus, the reaction with hydroxyl comprising compounds yield blocked isocyanates, which could be used to make useful derivatives. However, the yields are much less than with amines. Hence, in this thesis only compounds comprising amines are used as high yields were anticipated.

Here we describe the synthesis of caprolactam blocked lysine diisocyanates methyl ester (BLDI-OMe) and its derivatives, as building blocks for the preparation of bio-active polyurethanes. Conditions to modify independently either with the blocked isocyanate or with the methyl ester group will be discussed. The hydrolysis of the methyl ester, the

conversion of the acid into the NHS-activated ester and the further modifications thereof are reported.

2.2. Materials and Methods

2.2.1. Materials

CBC (carbonyl biscaprolactam), ALLINCO®, was kindly obtained from DSM Innovation Center, (> 99 % pure according to HPLC) and used without purification.
L-Lysine monohydrochloride, thionylchloride, calciumchloride dihydrate, hexyl amine, dibutyltin dilaurate (DBTDL, 95 %), 1-octanol, anhydrous magnesium sulfate, dicyclohexyl-carbodiimide (DCC), N-hydroxysuccinimide (NHS), chloroform-d ($CDCl_3$-D, 99.8 atom % D) and dimethyl sulfoxide-d_6 (DMSO-d_6, 99.5 atom % D) were purchased from Sigma-Aldrich.
Ethyl acetate, potassium hydroxide and anhydrous *N,N*-dimethylformamide (DMF), were purchased from Acros Organics.
Methanol, acetone, chloroform, triethylamine (TEA), n-hexane, hydrochloric acid fuming 37%, tetrahydrofuran (THF), dichloromethane (DCM), were purchased from Lab-Scan Analytical Science.

2.2.2. Analysis

Column chromatography was performed on silica gel (Silica-P flash silica gel from Silicycle, size 40-63 μm). TLC was performed on silica gel 60/Kieselguhr F254. Components were visualized by UV and staining with a solution of a mixture of $KMnO_4$ (5g) and K_2CO_3 (20 g) in H_2O (500 mL).
High performance liquid chromatography (HPLC) was carried out on a Shimadzu LC-10ADVP HPLC equipped with a Shimadzu SPD-M10AVP diode array detector. (non-chiral column, heptane/i-PrOH 95:5, gradient 30:70, 30 min, 0.5 mL / min, 254 nm).
Element analysis (EA) was performed on a HEKAtech Gmbh Euro-EA CHNanalyzer.
Mass spectra were recorded on a LTQ Orbitrap XL (ESI+).

^{1}H- and ^{13}C-NMR were recorded on a Varian AMX400 using $CDCl_3$ or d_6-DMSO as solvent. Chemical shift values are reported in ppm with the solvent resonance as the internal standard ($CHCl_3$: δ 7.26 for ^{1}H, δ 77.0 for ^{13}C; d_6-DMSO: δ 3.3 and 2.5 for ^{1}H, δ 39.5 and 40.5 for ^{13}C). Data are reported as follows: chemical shifts, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet).

2.2.3. Synthesis of blocked diisocyanates

2.2.3.1. Synthesis of lysine methylester dihydrochloride (1)[27]

Lysine monohydrochloride (78.59g, 0.43mol) were suspended in 600mL methanol, whilst being cooled at 0^{o}C by using an ice bath. Thionyl chloride (45mL, 0.62mol) was added drop wise whilst maintaining the temperature below 20^{o}C. The mixture was then refluxed for 18 hours at 63^{o}C under the nitrogen atmosphere. After the clear solution was cooled to room temperature within 4 hours, the L-lysine methylester dihydrochloride crystals precipitated and were isolated by filtration and washed with acetone. The filtrate was concentrated by evaporation and cooled in ice to form an additional quantity of crystals. This fraction was filtered as well and washed with acetone and added to the fraction crystals that had already been obtained. The combined product was dried at 50 ^{o}C for 48 hours in a vacuum oven, yielding a white powder product. The purity was over 99% based on HPLC, the yield 96.8%.

^{1}H-NMR (400 MHz, DMSO-d6): δ =1.38 to 1.61 (4H, m, $CH_2C\underline{H}_2C\underline{H}_2CH_2$), 1.84 (2H, m, $C\underline{H}_2CH$), 2.74 (2H, m, $NH_3ClC\underline{H}_2$), 3.75 (3H, s, $OC\underline{H}_3$), 3.97(1H, t, C$\underline{H}$), 8.29 (3H, s, $CH_2N\underline{H}_3Cl$), 8.82 (3H, s, $CHN\underline{H}_3Cl$).

2.2.3.2. Synthesis of caprolactam blocked lysine methylester diisocyanate (2) (BLDI-OMe)[27]

CBC (79.46g, 0.32mol) were dissolved in 150 mL chloroform at ambient temperature by stirring. To this solution L-lysine methylester dihydrochloride (36.63g, 0.16mol) were added. The resultant mixture was heated to 40 ^{o}C, after which 45mL (0.32mol)

triethylamine were added. The resultant mixture was refluxed at 76 ^{o}C for 60 hours in the nitrogen atmosphere. Next the mixture was cooled to ambient temperature within 1 hour, during which triethylamine hydrochloride crystals were formed. The crystals were removed by filtration. The filtrate was concentrated by evaporation to obtain viscous turbid orange oil liquid. This liquid was dissolved in 300mL of a mixture of ethyl acetate and hexane (2:1, v:v). The resultant solution was extracted with 400mL of a 0.5N HCl aqueous solution, which contained 5% (w/w) $CaCl_2$ and 5% (w/w) NaCl. Thereafter with 400mL 5% (w/w) $CaCl_2$ (aq), and subsequently with 400mL 1mol/L Na_2CO_3 (aq). The organic solution was died over anhydrous magnesium sulfate. After removing $MgSO_4$ by filtration, the solvent was removed by evaporation to yield a light yellow viscous oily substance in a yield of 97.5%.

Traces of CBC were removed by column chromatography with a mixture of hexane and THF (3:1, v: v). The compound was then dried in vacuum oven at 100^{o}C for 4 days to remove THF, yielding a colorless viscous oily product. The purity was over 99.5% according to HPLC measurements, and the yield was over 80%.

^{1}H-NMR (DMSO-d6): δ = 1.37 to 1.84 (18H, m, C$\underline{H}_2$C$\underline{H}_2$C$\underline{H}_2$ ring + CHC$\underline{H}_2$C$\underline{H}_2$C$\underline{H}_2$), 2.66 (4H, m, C$\underline{H}_2$CO), 3.22 (2H, m, NHC$\underline{H}_2$), 3.69 (3H, s, OC$\underline{H}_3$), 3.92 (4H, m, NC$\underline{H}_2$), 4.42 (1H, q, C$\underline{H}$), 9.21 (1H, t, CH_2N$\underline{H}$), 9.66 (1H, d, CHN$\underline{H}$).

^{13}C-NMR (DMSO-d6): δ = 179.94 (N$\underline{C}$OCH_2, ring), 172.77 (CH$\underline{C}$OOCH_3), 154.56 (NH$\underline{C}$ON), 52.53(COO$\underline{C}H_3$).

ESI-MS m/z: $(M+1)^+$ 439.25, $(M+Na)^+$ 461.24

EA: Cal: N, 12.78%; C, 57.52%; H, 7.81%; Exp: N, 12.60%; C, 57.49%; H, 7.89%

2.2.4. Model reaction of BLDI-OMe with 1-octanol

A 100 mL glass flask was fitted with a reflux condenser, a nitrogen inlet and an outlet to a vacuum pump. After the flask was three times evacuated and refilled with nitrogen, to remove all oxygen, a solution of BLDI-OMe (2.0 g, 4.6mmol) and 1- octanol (2.4g,18.4mmol) in dry DMF (10 mL) was added and heated up to 125 ^{o}C with or without 50µl DBTDL (dibutyl tin dilaurate), while stirred for 72 hours. The solvent and excess 1-octanol were removed by evaporation and then the residue was dissolved in

20mL mixture of ethyl acetate and hexane (2:1, v:v). The resultant solution was washed with 100mL 5% (m/m) $CaCl_2$ (aq), thereafter with 100mL aqueous solution of Na_2CO_3 (1mol/l) and subsequently with 100mL distilled water. The organic solutions were died over anhydrous magnesium sulfate. After removal of $MgSO_4$ by filtration, the solvent was removed by evaporation to yield a light yellow wax like product (3).

^{1}H-NMR (d6-DMSO): δ = 0.81 (6H, m, $CH_2C\underline{H}_3$), 1.23 to 1.62 (30H, m, alkane chain), 2.94 (2H, m, $NHC\underline{H}_2$), 3.60 (3H, s, $OC\underline{H}_3$), 3.88 to 3.93 (5H, m, $C\underline{H}$+$C\underline{H}_2CO$), 6.96 (1H, t, $CH_2N\underline{H}$), 7.42 (1H, d, $CHN\underline{H}$).

2.2.5. Hydrolysis of BLDI-OMe to caprolactam blocked 1-lysine diisocyanate acid (BLDI-OH) (4)

BLDI-OMe (10g, 22.8mmol) was suspended in 30mL THF and 70mL H_2O. The pH of this solution was adjust to 13 and maintained at this value for 5 hours at room temperature by the continuous addition of an aqueous KOH solution (2mol/l). The reaction mixture was then acidified to around pH 4 with 4M HCl, forming a milk like suspension. The acid containing BLDI-OH solution was extracted by diethyl ether until there was no suspension formed when a new charge of acid was added. The resultant diethyl ether solution was dried over anhydrous $MgSO_4$. After removal of $MgSO_4$, the solvent was evaporated and the residue was dissolved in a mixture of chloroform and methanol (60:1). After flash chromatography with chloroform and methanol with ratio of 60:1, the elute was evaporated and the solid was died at 100^{o}C for 7days in the vacuum oven, giving 8g (80%) free acid BLDI-OH.

^{1}H-NMR ($CDCl_3$): δ = 1.34 to 1.87 (18H, m, $C\underline{H}_2C\underline{H}_2C\underline{H}_2$ring + $CHC\underline{H}_2C\underline{H}_2C\underline{H}_2$), 2.62 (4H, m, $C\underline{H}_2CO$), 3.18 (2H, m, $NHC\underline{H}_2$), 3.88 (4H, m, $NC\underline{H}_2$), 4.39 (1H, q, $C\underline{H}$), 9.21 (1H, t, $CH_2N\underline{H}$), 9.62 (1H, d, $CHN\underline{H}$), 10.25 (1H, s, $COO\underline{H}$).

^{13}C-NMR ($CDCl_3$): δ = 179.9 ($N\underline{C}OCH_2$, ring), 174.5 ($CH\underline{C}OOH$), 154.5 ($NH\underline{C}ON$)

ESI-MS m/z: $(M+1)^+$ 425.24, $(M+Na)^+$ 447.22

EA: Cal: N, 13.20%; C, 56.59%; H, 7.60%; Exp: N, 13.10%; C, 56.02%; H, 7.69%

2.2.6. Synthesis of BLDI-NHS (active ester (5))

BLDI-OH (10g, 23.6mmol) was dissolved in 100 methylene chloride. To this solution dry and finely powdered N-hydroxysuccinimide (NHS, 2.74g, 23.8 mmol, molar ratio 1.01 times of BLDI-OH) was added. The flask was cooled in an ice-water bath, after which dicyclohexyl carbodiimide (DCC, 4.88g, 23.7mmol) was added in a molar ratio of 1.005 times of BLDI-OH. The reaction mixture was stirred vigorously at 0 ^{o}C for 1 hour and at room temperature for 24 hours. After the removal of precipitated dicyclohexyl urea by filtration, the crude product was extracted with distilled water. The resultant was dried over anhydrous $MgSO_4$. After removal of $MgSO_4$ by filtration, the product was further purified by flash chromatography with chloroform and methanol with ratio of 60:1. The pure product was collected to give 8.5g (85%) BLDI-NHS.

^{1}H-NMR ($CDCl_3$): δ = 1.42 to 2.04 (18H, m, C$\underline{H}_2$C$\underline{H}_2$C$\underline{H}_2$ring + CHC$\underline{H}_2$C$\underline{H}_2$C$\underline{H}_2$), 2.67 to 2.82 (8H, m, C$\underline{H}_2$CO), 3.30 (2H, m, NHC$\underline{H}_2$), 3.98 (4H, m, NC$\underline{H}_2$), 4.80 (1H, q, C$\underline{H}$), 9.26 (1H, t, CH_2N$\underline{H}$), 9.81 (1H, d, CHN$\underline{H}$).

ESI-MS m/z: $(M+1)^+$ 522.25, $(M+Na)^+$ 544. 24.

EA: Cal : N, 13.43%; C, 55.27%; H, 6.76%; Exp: N, 13.21%; C, 55.54%; H, 6.82%.

2.2.7. Synthesis of BLDI-HA (6)

BLDI-NHS (10g, 19.2mmol) was dissolved in 100 mL methylene chloride. A large excess of hexylamine (6.4mL, 48mmol) was added. The reaction mixture was stirred at room temperature overnight. After removal of precipitated NHS salt by filtration, the solution was washed with 300mL 5% $NaHCO_3$ aqueous solution and with 300mL brine (saturated NaCl aqueous solution). The resultant solution was dried over anhydrous $MgSO_4$. After removal of $MgSO_4$ by filtration and solvent by evaporation, the crude product was collected. Then after flash chromatography with chloroform and methanol with ratio of 60:1, the pure product was collected to give 9g (90%) BLDI-HA.

^{1}H-NMR ($CDCl_3$): δ = 0.83 (3H, m, C$\underline{H}_3$), 1.06 to 1.92 (26H, m, C$\underline{H}_2$C$\underline{H}_2$C$\underline{H}_2$ring + chains), 2.68 (4H, t, C$\underline{H}_2$CO), 3.22 (4H, m, NHC$\underline{H}_2$), 3.94 (4H, m, NC$\underline{H}_2$), 4.23 (1H, q, C$\underline{H}$), 6.24 (1H, t, N$\underline{H}$C$_5$H$_{10}$), 9.24 (1H, t, CH_2N$\underline{H}$), 9.52 (1H, d, CHN$\underline{H}$).

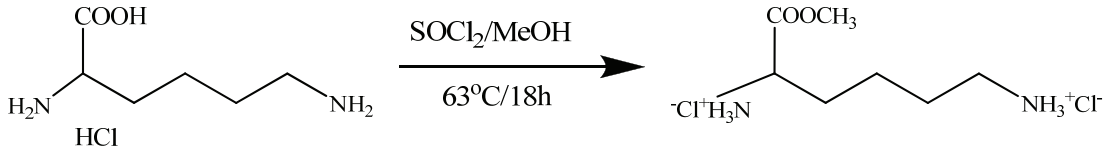
ESI-MS m/z: $(M+1)^+$ 508.35, $(M+Na)^+$ 530.33.

EA: Cal : N, 13.80%; C, 61.51%; H, 8.93%; Exp: N, 13.54%; C, 62.04%; H, 9.16%.

2.3. Results and discussion

2.3.1. Synthesis of blocked lysine diisocyanates

Polyurethanes are prepared from diisocyanates and polymeric diols. (Blocked) diisocyanates are synthesized from the corresponding diamines and phosgene. In cases where the polymers are used for implants, it is preferred to use a natural diamine, such as diamino butane, lysine or ornithine. The term "natural" is in particular used herein as being naturally present in a human body. Lysine is a natural diamine that has additionally an acid side group that could be very useful to incorporate bio-active moieties. Loontjens *et al.* showed that primary amines react quantitatively with carbonyl biscaprolactam (CBC), forming blocked isocyanates.[26] This route allows making blocked isocyanates without the need for making first the corresponding isocyanate. The reaction of CBC with the amino group of lysine, in which the acid group is on the α-position with respect to an amino group, was not studied yet. The steric and electronic influences of the acid (or ester) group nearby the amino group could hamper the reactivity severely. For making blocked diisocyanate this acid group had to be protected temporarily, as it could interfere during the synthesis. The protection group, however, should be removable in order to be able to couple later on bio-active molecules onto the acid side group. As shown in Scheme 2.4 a methyl ester was chosen for that purpose. It was expected that during the synthesis of the methyl ester with thionyl chloride the HCl-salt of lysine would be formed, which could prevent the reaction between the amino groups and the methyl ester.



Scheme 2.4 Preparation of the methyl ester of lysine as temporarily protection moiety of the acid group.

When lysine mono-HCl salt was mixed at 0°C in methanol with thionyl chloride, lysine acid chloride was formed. Subsequently, the acid chloride reacted with methanol under reflux to form methyl ester of lysine. After the clear solution was cooled to room temperature within 4 hours, the lysine methylester dihydrochloride crystals were isolated by filtration and washed with acetone. The white powder product was collected giving a yield of 96.8%. The purity of the crystals is over 99%, according to HPLC measurement. Figure 2.1 shows the ^{1}H-NMR spectrum of lysine methyl ester dihydrochloride (1). All signals in the spectra are in agreement with the expected product.

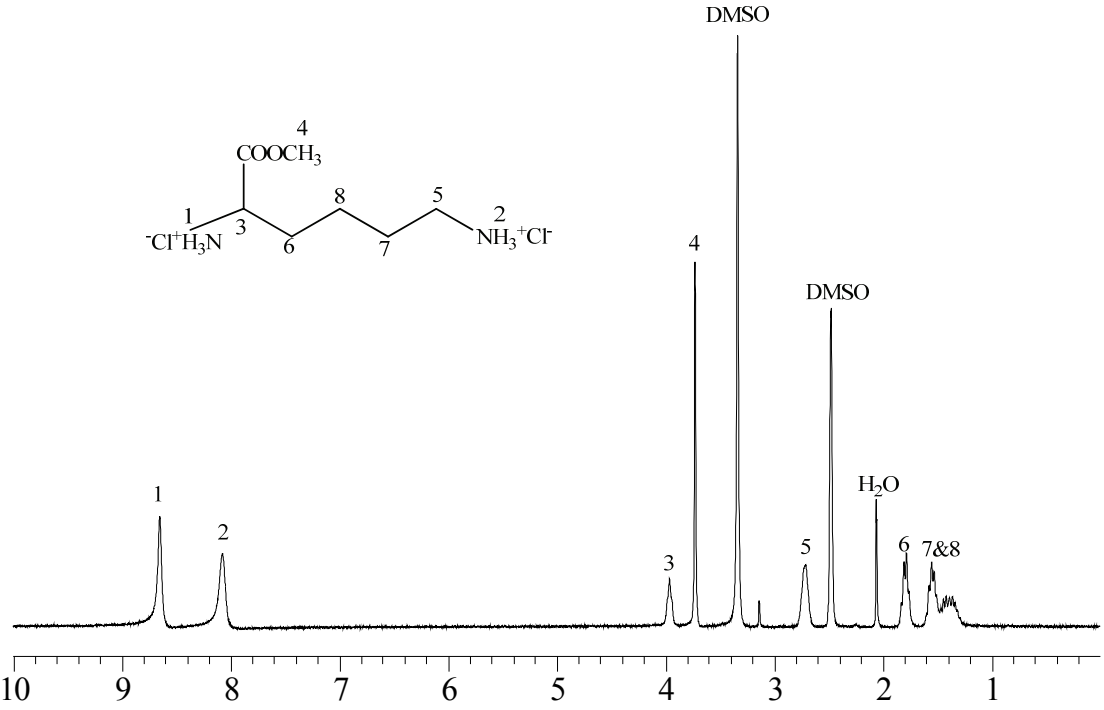
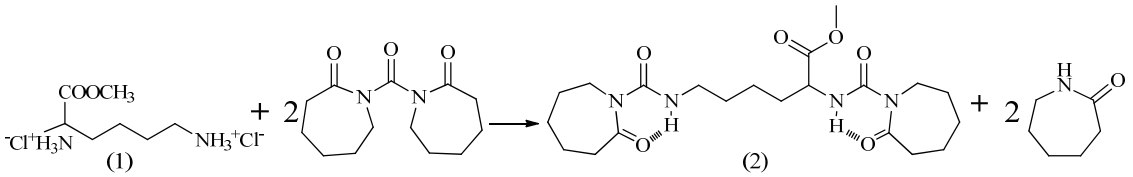


Figure 2.1 ^{1}H-NMR spectrum of lysine methylester dihydrochloride

After the protection step the caprolactam blocked lysine methylester diisocyanate (BLDI-OMe) was prepared according to the procedure as depicted in Scheme 2.5.



Scheme 2.5 Synthesis of caprolactam blocked lysine methyl ester diisocyanate

In a typical experiment a suspension of lysine methylester dihydrochloride in dry chloroform was added to a solution of CBC in chloroform. Triethyl amine (TEA) was added to scavenge HCl and to de-protonate the amino groups of lysine. During heating the formation of TEA-HCl salt can free the primary amine groups. The resulting mixture was refluxed at 76 ^{o}C for 60 hours in a nitrogen atmosphere. In contrast to the reaction with common primary amines, reaction times were now much longer. The primary amino group nearby the ester is indeed much less reactive (60h) than common aliphatic primary amines (8h). A slight excess of CBC with respect to the amino groups was used. It was found that the reaction of both primary amines with CBC went to completion when small excess CBC was used. Although the second caprolactam ring of CBC, *i.e.* the one of the blocked isocyanate, is substitutable as well, no such a reaction takes place below 100 ^{o}C. It was argued that the intermediate product is stabilized by intra-molecular hydrogen bonding of the –NH of the blocked isocyanate and the carbonyl group of caprolactam (Scheme 2.5), as evidenced by ^{1}H-NMR spectroscopy (NH at 9.21 and 9.66 respectively). Besides the desired blocked isocyanate, caprolactam and triethylamine hydrochloride crystals were formed. Most of the TEA-salt was removed by filtration. Caprolactam and residual TEA·HCl salt were readily water soluble, while the product was not. In that case a simple aqueous extraction was sufficient to separate caprolactam and TEA·HCl from reaction product. The addition of $CaCl_2$, NaCl and HCl increases the solubility of caprolactam in water and decrease the solubility of blocked diisocyanate at same time, thereby improving the purification procedure. Hence, this method is very suitable to make lysine blocked isocyanates in high yield. Although the purity of the compound was already quite high, the monomer was still purified by column chromatography to be on the safe side for the consecutive polymerizations.

Figure 2.2 shows the ^{1}H-NMR spectrum of blocked lysine diisocyanate methyl ester (BLDI-OMe) (2) before and after the column chromatography purification step. All

signals in the spectra were in agreement with the expected product, whereas only small traces of impurities are visible. The signals of CBC (e.g. at 3.86ppm), and of the amines have completely disappeared, whereas the signals of released caprolactam (e.g. at 2.4ppm) is almost absent.

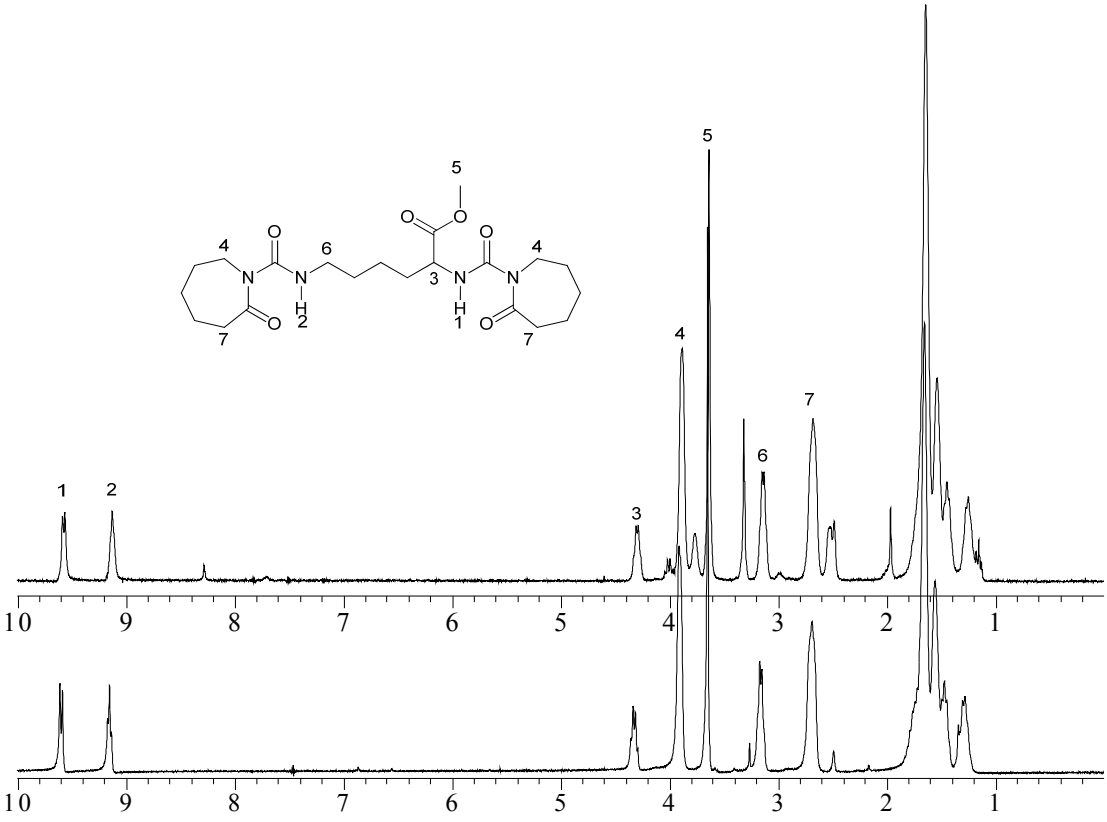


Figure 2.2 [1]H-NMR spectra of caprolactam blocked lysine diisocyanate methyl ester; top: before column chromatography; below: after column chromatography.

For sake of completeness, the ^{13}C-NMR spectrum of the purified monomer is given as well (Figure 2.3), although they cannot be used for quantitative analysis. The signals in the ^{13}C-NMR spectra were in agreement with the expected products. The signals of CBC (e.g. at 176 ppm and 157 ppm) and of the amines have completely disappeared, whereas the signal of the released caprolactam (e.g. at 178 ppm) is almost invisible.

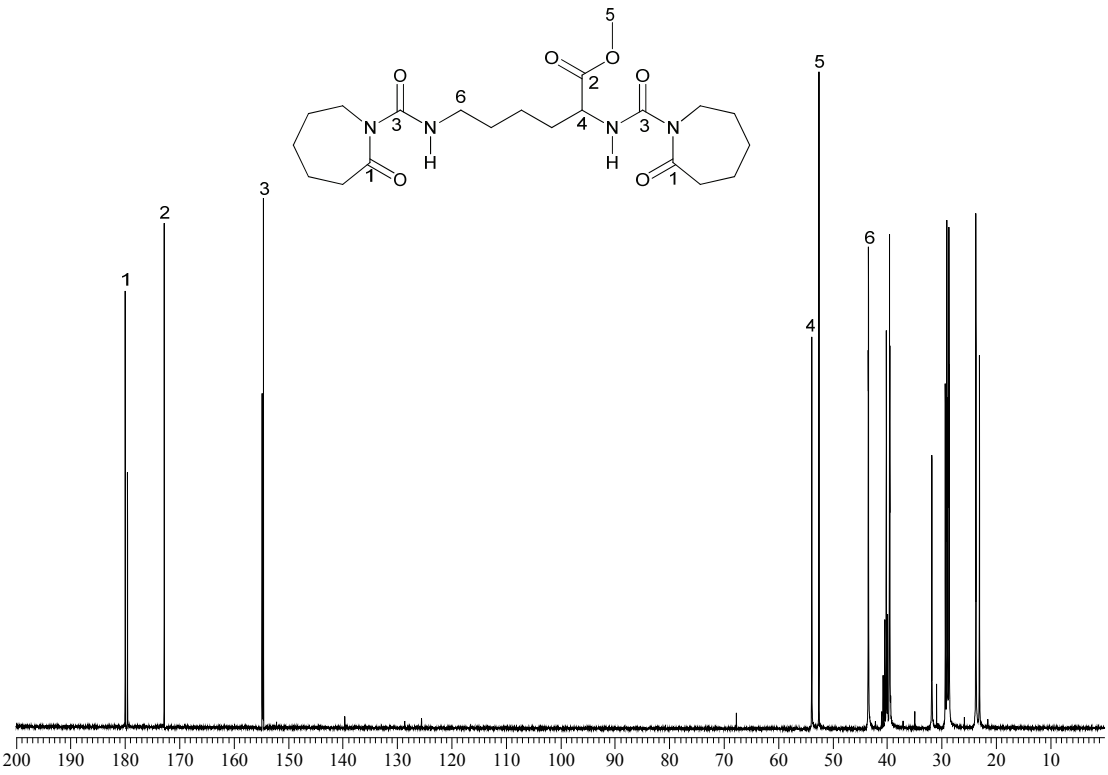


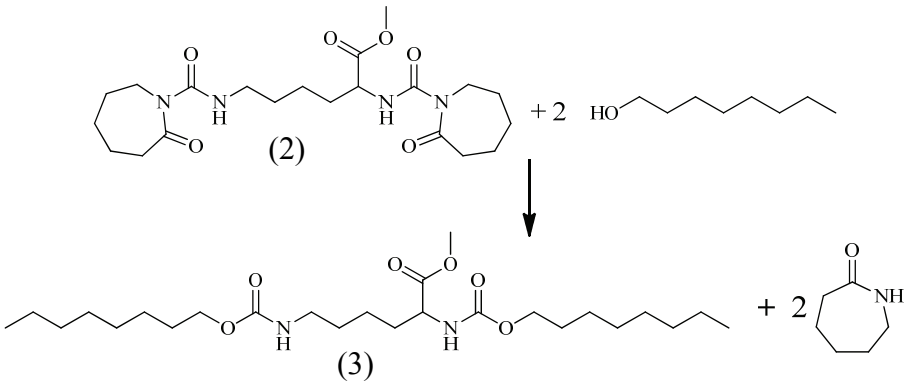
Figure 2.3 ^{13}C-NMR spectrum of caprolactam blocked lysine diisocyanate methylester (2).

Besides NMR techniques, the product was also analyzed by ESI-MS and elemental analysis, as well as by high performance liquid chromatography (HPLC). Based on all these techniques the purity of product appeared to be over 99%. Furthermore, because of strong hydrogen bonding, the product is very stable. After storage for three years no change was noticed by ^{1}H-NMR. Compound (2) comprised two blocked isocyanates and one methyl ester functional groups. The ester group was intended to be used as an anchor group for further modifications, whereas the blocked isocyanates were meant to make polyurethanes. Hence, it was essential for this work that both active groups could be activated separately.

2.3.2 Model reaction of the blocked diisocyanate with 1-octanol

The polyaddition reaction of polyols onto diisocyanates is the common route to make polyurethanes. Yet, it is also possible to react blocked diisocyanates with polydiols,

forming the same polyurethanes. However, blocked isocyanates are less reactive and higher temperatures are needed for the polymerization. The pendant ester functionality is in principle accessible for transesterification reaction, which would result in branched or even crosslinked products. Hence, it was important to find the conditions to substitute selectively caprolactam by a hydroxyl comprising compound, without attacking the methyl ester side group. To study this reaction in detail 1-octanol was chosen as model compound. The obtained low molecular weight products were better analyzable and the excess of octanol could easily be removed under vacuum. The model reaction was studied with and without dibutyltin dilaurate (DBTDL), a common catalyst for the preparation of polyurethanes. The reactions were followed by ^{1}H-NMR. The deblocking temperature of caprolactam blocked diisocyanate in the presence of hydroxyl functional compounds starts at about 125 ^{o}C. In order to try to suppress or even prevent the participation of the methyl ester group, a reaction temperature of 125^{o}C was applied. The reaction is depicted in Scheme 2.6.



Scheme 2.6 Modeling reaction of BLDI-OMe and 1-octanol

During the reaction the caprolactam rings were substituted by 1-octanol forming urethane linkages. The formed caprolactam was easily removed by the aqueous washing steps.

We found that, in the presence of dibutyl tin dilaurate (DBTDL), not only the blocked isocyanates, but also the ester reacted to a large extend with octanol, *i.e.* yielding branched products. In Figure 2.4 it can be seen that the sharp singlet of the methyl ester

peak at 3.6ppm (orange square) is strongly reduced. As a result the reaction of BLDI-OMe with diols in the presence of DBTDL would lead to crosslinked products.

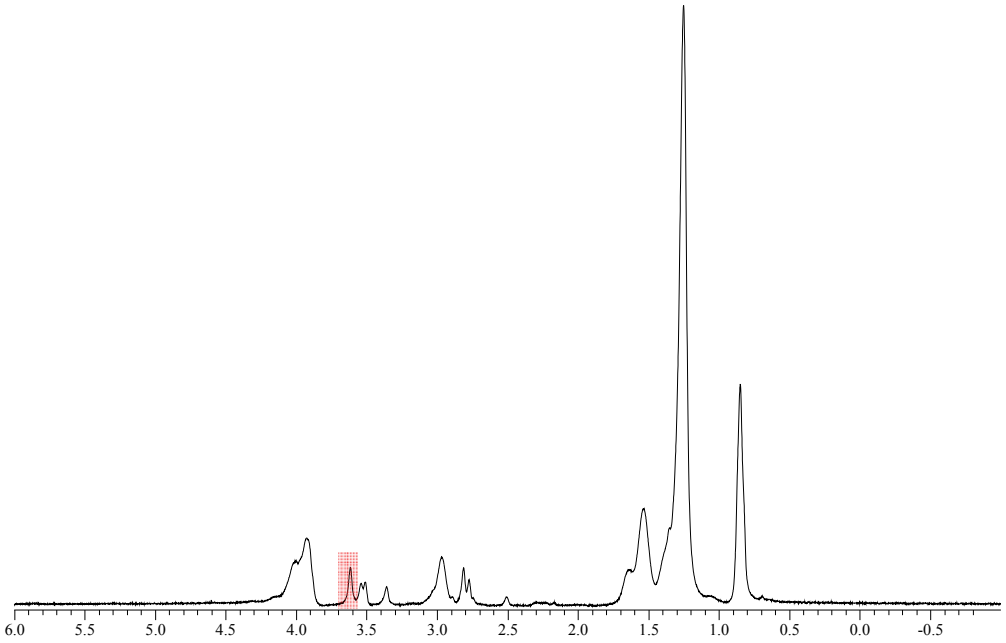


Figure 2.4 ^{1}H-NMR spectrum of modeling reaction of BLDI-OMe and Octanol with catalyst DBTDL.

Fortunately, the desired product was obtained in high yields when the reaction was performed without the tin catalyst (DBTDL). In Figure 2.5 the ^{1}H-NMR spectrum is given of resulting product obtained from the reaction of BLDI-OMe with an excess of 1-octanol without catalyst.

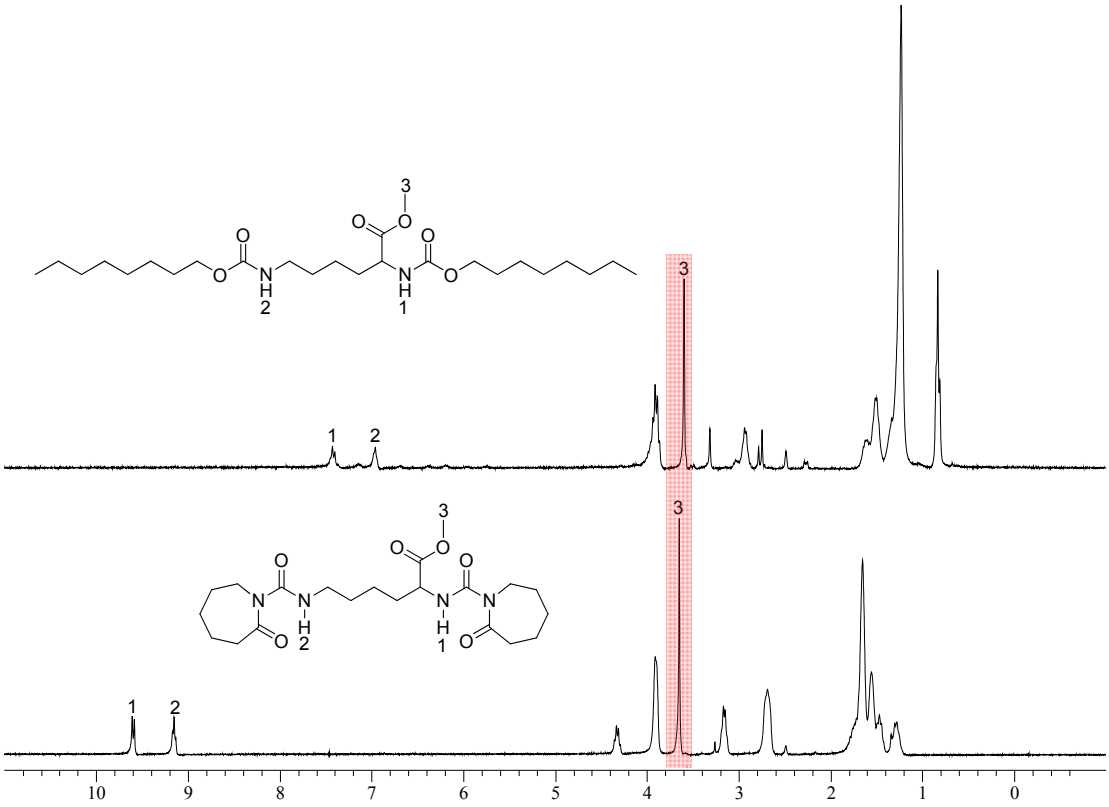
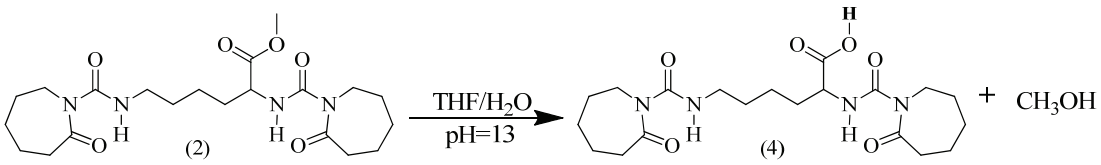


Figure 2.5 [1]H-NMR spectra of caprolactam blocked lysine diisocyanate (BLID-OMe) and the reaction product of BLDI-OMe and octanol, without using a catalyst.

The NH signals of the caprolactam blocked isocyanate moieties with their characteristic absorption at 9.66 and 9.21 ppm were absent after the reaction. Instead two new NH signals appeared at 7.42 and 6.92 ppm, which were assigned to the urethane protons. These NH signals shift to higher field is due to the absence of intramolecular-hydrogen bonding which was induced by caprolactam ring. More importantly, the signal of methyl ester $COOCH_3$ (orange square) was still there and was even completely maintained. This means that the methyl ester anchor group survived 72 hours at 125^{o}C heating in the presence of an excess of octanol, which is very important for preparing polyurethanes with pendant ester groups, needed for further modification of polymers. ^{13}C-NMR showed the absence of carbonyl group (179.9 ppm) of caprolactam ring, further supporting the complete progress of the reaction.

2.3.3 Hydrolysis of BLDI-OMe yielding caprolactam blocked lysine diisocyanate acid (BLDI-OH) (4)

To enable the necessary derivatization reactions, reaction conditions had to be found to hydrolyze the methyl ester side chains without hydrolyzing the blocked isocyanate groups. Hydrolysis of the ester had to be done before the polymerization, since the ester linkages of the polyester diols in the polyurethane backbone are susceptible to cleavage as well. We found finally conditions under which the ester of BLDI-OMe (2) can be hydrolyzed without hydrolyzing the blocked diisocyanate groups. The reaction was performed at room temperature at pH 13 (Scheme 2.7).



Scheme 2.7 Hydrolysis of the methyl ester of BLDI-OMe

The reaction was followed by monitoring in the ^{1}H-NMR spectrum the disappearance of CH_3 signal of methyl ester side chain and the appearance of the free carboxylic acid signal at 3.60 ppm and 10.25 ppm respectively (Figure 2.6). The reaction was complete after about 5 h. All signals in the spectrum can be assigned to the expected products. ESI-MS and elemental analysis were supporting evidence that the desired product was obtained.

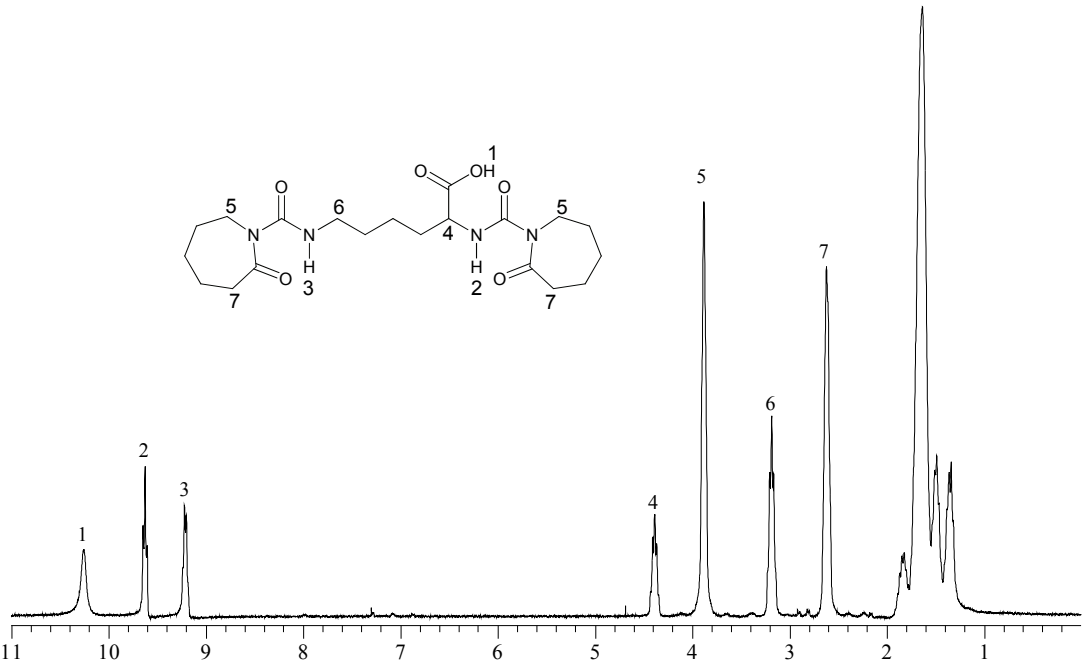
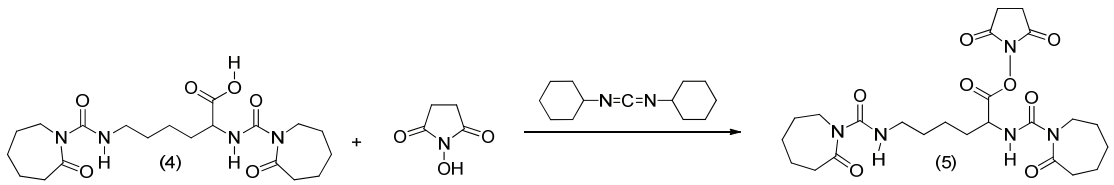


Figure 2.6 ^{1}H-NMR spectrum of hydrolyzed blocked diisocyanate BLDI-OH.

2.3.4. Synthesis of BLDI-NHS (5) (active ester)

The pendant carboxylic acids can used to tether functional groups. However, harsh reaction conditions to modify carboxylic acids were not preferred. A more general method is to use activated esters,[28-30] which react under ambient conditions. There were several options to convert the free acid of blocked diisocyanate into the active ester. One option was to react free acid with the N-hydroxysuccinimide (NHS) to form NHS activated ester. This activated ester can easily react with all kind of nucleophiles, such as the N-terminus of the peptide or proteins, forming a stable covalent amide bond.

To facilitate the reaction with N-hydroxy succinimide dicyclohexyl-carbodiimide (DCC) was used to activate carboxylic acid group (Scheme 2.8).



Scheme 2.8 Synthesis of NHS active ester caprolactam blocked lysine diisocyanate BLDI-NHS

The reaction was performed at room temperature and monitored, by ^{1}H-NMR, the disappearance of COOH signal and appearance of NHS signal at 10.25 ppm and 2.82 ppm respectively (Figure 2.7). The reaction completed in high yields after about 24 h. All signals in the spectrum could be assigned to the expected products.

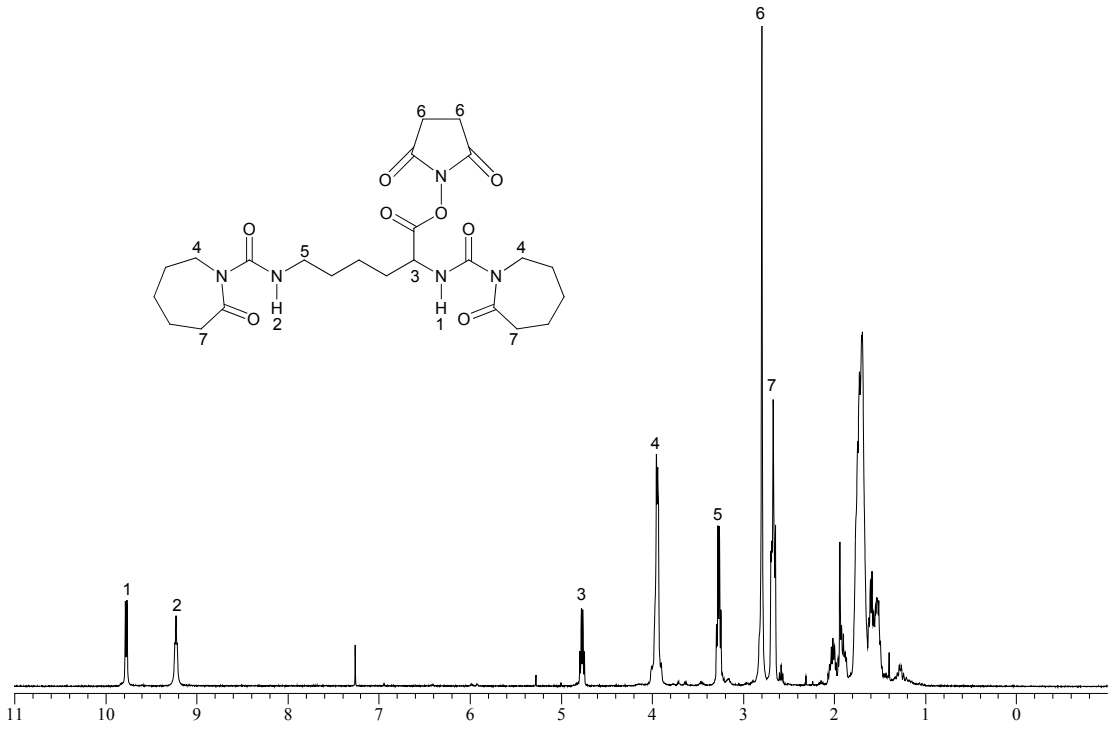
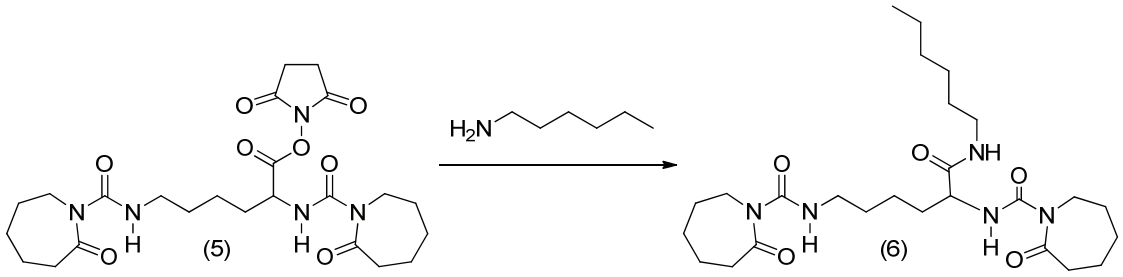


Figure 2.7 ^{1}H-NMR spectrum of NHS active ester caprolactam blocked lysine diisocyanats BLDI-NHS

ESI-MS and elemental analysis were supporting evidence that the desired product was obtained. Although NHS active esters are reactive, they appeared to be stable enough to be stored under dry conditions for several months without any sign of deterioration.[31,32]

2.3.5. Synthesis of BLDI-HA (6) for biomaterials modeling reaction

A final prove of the feasibility of our route to make lysine blocked diisocyanate with pendant functional groups was to substitute the NHS group by a simple amine, for which hexyl amine was used. To prevent hydrolysis of NHS by traces of water[29] anhydrous methylene chloride was used as solvent. Hexylamine substituted indeed the NHS group, without affecting the blocked isocyanate groups. While being coupled onto the blocked diisocyanate BLDI-HA (6) was formed and the precipitation of the NHS-amine salt was noticed at same time (Scheme 2.8).



Scheme 2.8 Synthesis of hexylamine caprolactam blocked lysine diisocyanate BLDI-HA

The reaction was performed at room temperature and monitored by 1H-NMR via the disappearance of NHS signal and appearance of amide and CH_3 of hexylamine signals at 2.82, 6.38 and 0.8 ppm respectively (Figure 2.8). The reaction was complete after about 24 h. All other signals in the spectrum could be assigned to the expected products. ESI-MS and elemental analysis supported furthermore that the desired product was obtained. The coupling reaction, which was successfully performed in high yields, could be seen as a model reaction for coupling of the N-terminus of peptides, proteins or other bio-active compounds.

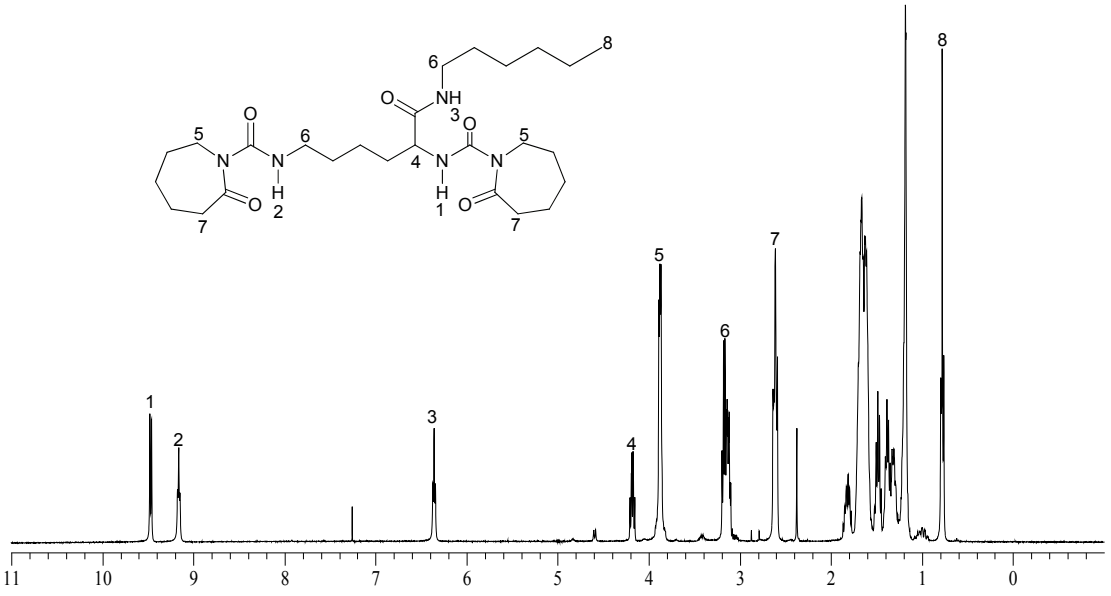


Figure 2.8 ^{1}*H-NMR spectrum of hexylamine caprolactam blocked lysine diisocyanate BLDI-HA*

2.4. Conclusion

The lysine blocked diisocyanate methyl ester was successfully synthesized in high yield by reacting the methyl ester of lysine HCl-salt, in the presence of a base, with carbonyl biscaprolactam (CBC). In a highly selective step only one of the two caprolactam rings of CBC was substituted by the primary amines, yielding the methyl ester of caprolactam blocked lysine diisocyanate. The caprolactam blocked lysine diisocyanate groups reacted exclusively with 1-octanol in high conversions without modifying the methyl ester, but only when no metal catalyst was used. This offered probably enabling conditions to make linear polyurethanes with pendant ester groups.

In order being able to introduce pendant bio-functional side groups we found the condition at which we could hydrolyze the ester group without affecting the blocked isocyanate groups. This is the major advantage of blocked diisocyanate over the methyl ester of lysine diisocyanate.

The free acid group was converted into the N-hydroxysuccinimide activated ester. The activated ester allows modification with various nucleophiles. In a model reaction the substitution of the NHS-group by hexyl amine was successfully performed.

The various modified caprolactam blocked lysine diisocyanates can be used to make very well defined polymer structures, which will be discussed in the following chapters.

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Chapter 3

Linear polyurethanes based on caprolactam blocked lysine diisocyanate methyl ester

Abstract

The aim of this chapter was to make linear polyurethanes by using the difference in reactivity of the blocked isocyanate groups and the methyl ester of caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe) in the reaction with polydiols. For that poly(ε-caprolactone) (PCL) and poly(ethyleneglycol) (PEG) were selected as representative examples for the two classes of polyurethanes (PUs). Poly(ester-urethanes) were successfully synthesized by reacting PCL with BLDI-OMe, in solution as well as in bulk. In bulk the polymerizations were much faster than in solution. Above 180 ^{o}C, in bulk, branching of the polyurethanes happened. As a result, depending on the conditions, either linear or branched polymers or even polymer networks could be made with the same monomer mixtures.

Polyethylene glycol was selected to study the polymerization performance of polyethers with BLDI-OMe in bulk. In contrast to PCL, linear poly(ether-urethane) could not be obtained in this case. Even at 125^{o}C (deblocking temperature of caprolactam blocked isocyanate group), the reactivity of hydroxyl groups of PEG towards the ester group was already too high. As a result, poly (ether-urethane) networks were formed after prolonged heating. This offered a convenient way to make polyurethane networks, which will be discussed in chapter 4.

3.1. Introduction

As described in Chapter 1, polyurethanes (PUs) have been widely used in biomedical devises. PUs are highly biocompatible and have widely tunable properties. Commonly they are prepared from diisocyanates and polymeric diols. However, the same polymers can be prepared by using blocked diisocyanates instead of unprotected isocyanates. The only difference is that the polymerization rate is slower due to the protective group. Above a certain threshold temperature hydroxyl groups are able to substitute the blocking group, yielding polyurethanes. The advantage of blocked isocyanates is that they are stable below 100 ^{o}C, allowing chemical modifications, which are impossible with unprotected isocyanates.

Numerous groups, including ours, have investigated biodegradable linear polyurethanes comprising degradable polyester as soft segments, and various diisocyanates and chain extenders as hard segments.[1-12] Varying the chain extender to diisocyanate ratio in the hard block, or altering the molecular weight or composition of the polyester in the soft block will dictate the ultimate physical properties of the polymers and the ultimate degradation rate.[3-8]

During degradation of polyurethanes formations of carcinogenic and mutagenic aromatic diamines have been reported, originating from the aromatic diisocyanates. Whether the concentration of these harmful degradation products attain a physiologically relevant level is still unresolved and in debate.[13,14] An alternative for aromatic isocyanates, in order to eliminate the concern of aromatic amines, is the utilization of diisocyanates based on amino acids, such as lysine.[15-17] These compounds provide a route to synthesizing biodegradable polyurethanes that are expected to yield only non-toxic degradation products. Bruin *et al*. reported that if the diisocyanates were liberated by hydrolysis of the urethane bonds of the polymer during degradation, the isocyanate functionalities would react with water to regenerate the diamine. If lysine diisocyanate ethyl ester was used , an essentially nontoxic diamine would be formed.[18] Subcutaneous implantations in guinea pigs elicited what the authors described as ‘‘no adverse tissue reactions”.[19] Other researchers have utilized lysine diisocyanate (LDI) and butane diisocyanate (BDI) to develop both crosslinked and linear biodegradable polyurethane

elastomers for biomedical applications and have not described any significant toxic or tumorigenic responses of these materials upon implantation.[20–25] Also, BDI- and LDI-based polyurethanes have been reported to be both tissue and cell biocompatible .[22-27]

So far, only the non-blocked lysine diisocyanate was used in biomedical polyurethanes. The use of free isocyanates has some serious drawbacks. Remnant traces of the high reactive and highly toxic isocyanates in the human body are not acceptable. More importantly, the presence of the isocyanate group did not tolerate any chemical modification of the ester side group of lysine. Hence, introduction of relevant bio-active functional groups via this ester function was not feasible.

As described in Chapter 2, the caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe) reacted exclusively with 1-octanol in high conversions via the blocked isocyanate groups without affecting the ester. It appeared to be essential that no catalyst was used. This offered perhaps the possibility to react functionalized blocked lysine diisocyanate with diols, affording catalyst-free linear polyurethanes with functional side groups.

In this chapter, the synthesis is reported of polyurethanes (PUs) that were obtained from blocked lysine diisocyanate (prepared according to the method as described in Chapter 2) and polycaprolactone and poly(ethylene glycol) diols. The synthesis, characterizations and properties of the PUs are described in detail.

3.2. Materials and Methods

3.2.1. Materials

CBC (carbonyl biscaprolactam) was obtained from DSM Innovation Center, ALLINCO®, (> 99 % pure according to HPLC) and used without purification.

L-Lysine monohydrochloride, thionylchloride, calciumchloride dihydrate, anhydrous magnesium sulfate, poly(ethyleneglycol)600 (PEG600), poly(ε-caprolactone)530 (PCL530), chloroform-d ($CDCl_3$-D, 99.8 atom % D) and dimethyl sulfoxide-d_6 (DMSO-d_6, 99.5 atom % D) were purchased from Sigma-Aldrich.

Ethyl acetate and anhydrous N,N-dimethylformamide (DMF), were purchased from Acros Organics.

Methanol, acetone, chloroform, triethylamine (TEA), n-hexane, hydrochloric acid fuming 37%, tetrahydrofuran (THF), were purchased from Lab-Scan Analytical Science.

Caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe) was synthesized according to the method as described previously (chapter 2).

3.2.2. Analysis

Column chromatography was performed on silica gel (Silica-P flash silica gel from Silicycle, size 40-63 μm). TLC was performed on silica gel 60/Kieselguhr F254. Components were visualized by UV and staining with a solution of a mixture of $KMnO_4$ (5g) and K_2CO_3 (20 g) in H_2O (500 mL).

Size exclusion chromatography (SEC) measurements were performed in dimethylformamide with 0.01M LiBr on a Viscotek GPC max equipped with model 302 TDA detectors, using 2 columns (Pl-gel 5μ 30 cm mixed-C from Polymer Laboratories). The data analysis was done using conventional calibration with polystyrene standards accompanied by in-house software.

^{1}H-NMR spectra were recorded on a Varian AMX400, using $CDCl_3$ as solvent.

ATR-FTIR was done using broker IFS88 spectrometer equipped with a Golden Gate (Graseby specac) single reflection ATR accessory. Spectra resolution was 4 cm^{-1} and 32 scans were taken per spectrum

Differential Scanning Calorimetry (DSC) measurements were performed on a Perkin Elmer DSC 7 instrument. The samples with masses varying between 7-10 mg were heated from -60 ^oC to +90 ^oC with a rate of 10 ^oC / min.

3.2.3. Solution polymerization of BLDI-OMe and PCL530

3.2.3.1 Optimizing the ratios between PCL530 and BLDI-OMe

A 100 mL glass flask was fitted with a nitrogen inlet and an outlet to a vacuum pump. After the flask was evacuated and refilled with nitrogen for three times, to remove all

oxygen, a solution of BLDI-OMe (2.0 g, 4.6mmol) and various amounts of PCL530 in the anhydrous DMF (10 mL) was added and heated to 125 ^{o}C while stirring. After 72 hours, samples were taken and the polymer solutions were poured into the diethyl ether. The precipitate was separated by centrifugation and, after the supernatant was removed, the polymers were dried in a vacuum oven at 50 ^{o}C for 48 h.

3.2.3.2. Polymerization kinetics

A 100 mL glass flask was fitted with a nitrogen inlet and an outlet to a vacuum pump. After the flask was three times evacuated and refilled with nitrogen, to remove all oxygen, a solution of BLDI-OMe (2.0g, 4.6mmol) and PCL530 (2.38g, 4.5mmol) in the anhydrous DMF (10 mL) was added and heated up to 125 ^{o}C while stirring, for several days. During the polymerization, after various time intervals, samples were taken and poured into the diethyl ether. The precipitate was separated by centrifugation and after the supernatant was removed the polymers were dried in a vacuum oven at 50 ^{o}C for 48 h. The molecular weights were determined by size exclusion chromatography (SEC).

3.2.4. Bulk polymerization of BLDI-OMe and PCL530

3.2.4.1 Polymerization kinetics

A 100 mL glass flask was fitted with a nitrogen inlet and an outlet to a vacuum pump. After the flask was three times evacuated and refilled with nitrogen, to remove all oxygen, dry BLDI-OMe and PCL530 were added in a molar ratio of 1.025. The mixture was heated up to 125 ^{o}C while stirring under vacuum, for several days. Samples were taken at several time intervals and analyzed by SEC measurement directly, without purification by precipitation.

3.2.4.2. Polymerization at higher temperatures in bulk

A 100 mL glass flask was fitted with a nitrogen inlet and an outlet to a vacuum pump. After the flask was three times evacuated and refilled with nitrogen, to remove all oxygen, dry BLDI-OMe and PCL530 were added in a molar ratio of 1.025. The mixture was subsequently heated up to 145 ^{o}C, 160 ^{o}C, 180 ^{o}C respectively, while stirring under

vacuum, for various time till 24 hours. Samples were taken and analyzed by SEC measurement directly, without purification by precipitation.

3.2.5. Bulk polymerization of BLDI-OMe and PEG600

A 100 mL glass flask was fitted with a nitrogen inlet and an outlet to a vacuum pump. After the flask was three times evacuated and refilled with nitrogen, to remove all oxygen, dry BLDI-OMe and PEG600 were added in a 1:1 molar ratio. The mixture was heated up to 125 ^{o}C while stirring under vacuum for 72hours. Samples were taken and analyzed by SEC measurement directly, without purification by precipitation.

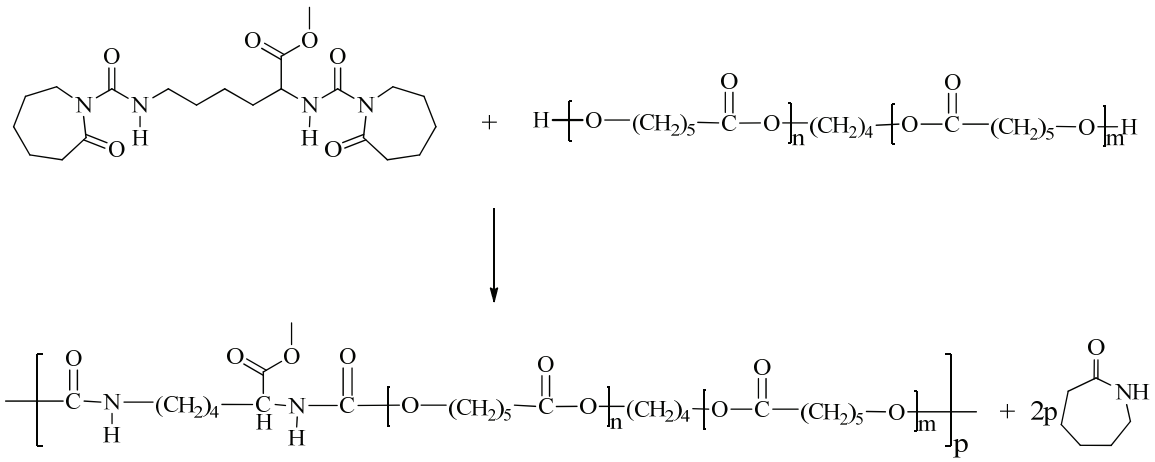
3.3. Results and discussion

3.3.1. Solution polymerization of BLDI-OMe and PCL530

As described in Chapter 2, 1-octanol reacted exclusively and in high conversions with the blocked isocyanate groups of caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe) and not with the ester moiety, but only when no catalyst was used. Hence, linear polyurethanes could be expected to be obtained when polydiols were used instead of 1-ocatanol. The most commonly used polydiols for synthesizing polyurethanes are polyester and polyether diols. Here, poly(ε-caprolactone)530 (PCL530) and poly(ethylene glycol)600 (PEG600) were used to represent polyester and polyether diols. Poly (ε-caprolactone) is commonly used as soft segment polyester in polyurethanes. It is known to be biocompatible, slowly hydrolysable and enzymatically degradable. The molar weight of linear polyurethanes has to be relatively high, having a sufficient quantity of the crystalline hard phase to increase physical crosslinking, in order to achieve good mechanical properties. However, residues of the crystalline fraction caused inflammatory reactions *in vivo*.[28, 29] Here we chose therefore a low molecular weight PCL530 as soft segment to react with blocked lysine diisocyanate, forming amorphous polyurethanes. Due to the asymmetrical structure of lysine diisocyanate it is difficult, maybe even impossible to obtain crystalline hard segments. Moreover, the pendant ester

side group offered the possibility to form chemical instead of physical crosslinks, which enables to tune the mechanical properties of these amorphous polymers.

Initially, the polymerizations were done by heating a solution of BLDI-OMe and PCL530 in various ratios in anhydrous DMF in a nitrogen atmosphere at 125 ^{o}C, which is above the deblocking temperature of caprolactam blocked isocyanates.[30,31] The polymerization scheme is depicted in Scheme 3.1. During the polymerization the caprolactam rings were substituted by PCL530, forming the corresponding polyurethanes. Samples were taken after 72 hours and were precipitated in diethyl ether to remove caprolactam and low molecular compounds. The polymers were collected by centrifugation and, after the supernatant was removed by decantation, the samples were dried in an oven before performing the molecular weights analysis by SEC.



Scheme 3.1 Synthesis of polyurethanes from the blocked lysine diisocyanate (BLDI-OMe) and poly(ε-caprolactone) (PCL), polymerized without catalyst .

Synthesis of PUs is a typical condensation polymerization. The stoichiometry of polymerization reactants is very important for getting high molecular weights.[32] Therefore, polymerizations with various ratios between the amounts of blocked diisocyanate and PCL530 were performed to equalize the molar ratio of the functional groups. The molecular weight of the commercial PCL530 may be different from exactly

530 Da. Hence, the optimal weight ratios have to found by trial and error, near the stoichiometric point. All the PUs prepared in DMF at 125 ^{o}C were well soluble in DMF, even at room temperature, without any sign of gel formation. Since the polymers dissolved well in DMF the SEC analyses were therefore performed in the same solvent. Table 3.1 gives the molecular weights of PUs, which were obtained by varying the ratio between BLDI-OMe and PCl530. From the table, it can be seen at a molar ratio of 1.025 between blocked diisocyanate and PCL530, thus with a small excess of the BLDI-OMe, the highest molecular weight was obtained. This means that the molecular weight of PCL530 was very close to its theoretical value, assuming that the purity of BLDI-OMe was high. The PDI of all polymers was below 2, meaning that no branching took place under those conditions. So, the molar ratio as found in this series of experiments was also chosen in the next series of polymerizations.

Table 3.1 Molecular weights of PUs depending on the theoretical molar ratio between BLDI-OMe and PCl530 (72h, 125 ^{o}C).

Ratio of BLDI-OMe : PCl530	M_n (Da)	M_w (Da)	P.D.I
1.200 : 1	5,100	6,600	1.3
1.102: 1	5,700	7,500	1.3
1.074 : 1	5,800	8,200	1.4
1.050 : 1	6,400	7,400	1.2
1.025 : 1	7,500	13,900	1.8
1.003 : 1	6,800	11,700	1.7

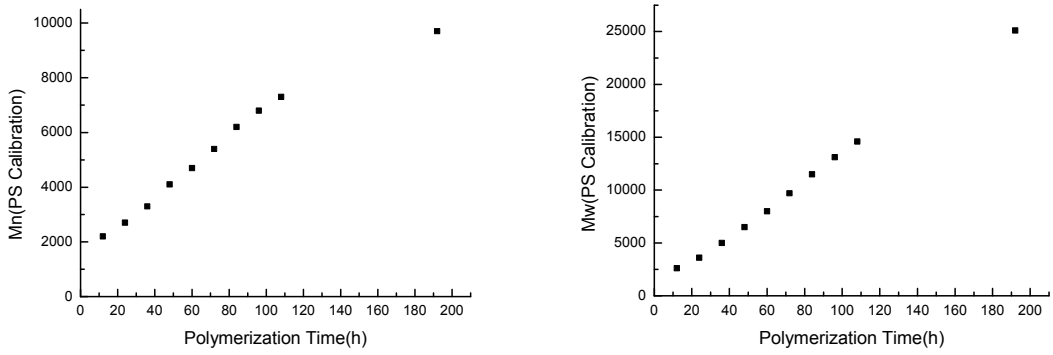
The molecular weight of polymers obtained from condensation polymerizations, also named step-growth polymerizations, increases step by step till one kind of functional groups is totally consumed.[32] Therefore, the optimization of the stoichiometry was important.

After having established the optimal molar ratio, the polymerization kinetics of PUs from BLDI-OMe and PCL530 were studied. During the polymerization at 125 ^{o}C in DMF, every 12 hours samples were taken, until 192 hours and the molecular weights were measured by SEC. From table 3.2 and figure 3.1 it can be seen that during the

polymerization until 192 hours, the number average molecular weight (M_n) increased in time to about 10,000 Da. The polydispersity was low (< 2.0) until a polymerization time of 108h (M_n = 7,300 Da) and became somewhat larger (2.6) at the end of the polymerization (192h), but the PDI was still narrow for condensation polymerizations that could potentially give branched polymers. The PDI value shows that, at least until a M_n of 7,300 Da, linear polymers were obtained. If prolonged reaction times were applied higher molecular weight polymers were obtained, and it appeared that some branching might have occurred.

Table 3.2 SEC results of polyurethane molecular weights depend on polymerization time, in DMF as solvent

Time(h)	12	24	36	48	60	72	84	96	108	192
M_n (Da)	2,200	2,700	3,300	4,100	4,700	5,400	6,200	6,800	7,300	9,700
M_w (Da)	2,600	3,600	5,000	6,500	8,000	9,700	11,500	13,100	14,600	25,100
PDI	1.19	1.34	1.49	1.60	1.69	1.81	1.85	1.93	2.00	2.58



(a) **(b)**

Figure 3.1 The relationship between molecular weights and reaction time of solution polymerization of PCL and BLDI-OMe in DMF at 125°C: (a) M_n vs time, (b) M_w vs time.

In Figure 3.2 the ^{1}H-NMR spectrum is given of the polyurethane with a M_n of 9,700 Da and a DPI of 2.58. All signals in the spectrum were in agreement with the expected products, originating from lysine diisocyanate and PCL530. Importantly, the sharp singlet

signal of methyl ester $COOC\underline{H}_3$ (3.5ppm) is still there and is even completely maintained, according to NMR. It means that the pendant methyl ester group did not react at all with hydroxyl groups at 125 ^oC, which confirms the model reaction in chapter 2 (scheme 2.6).

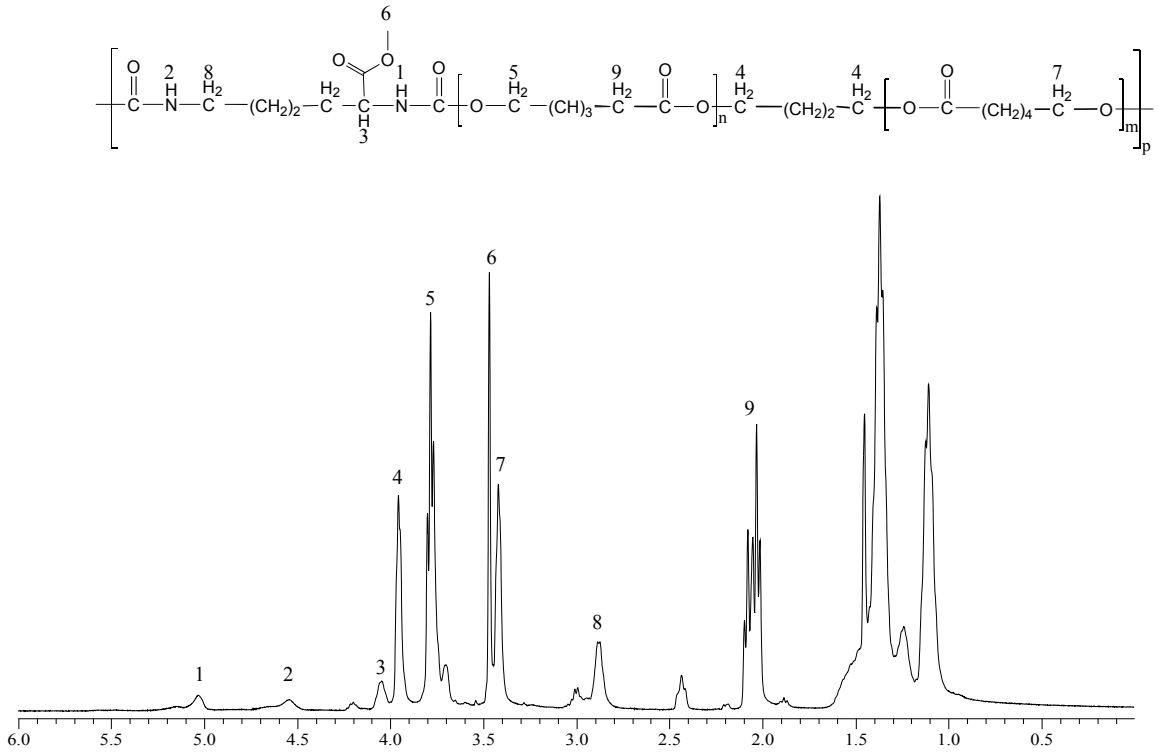


Figure 3.2 ^{1}H-NMR spectrum ($CDCl_3$) of resulting product obtained from the solution reaction from BLDI-OMe and PCl530 which M_n and DPI are 9,700 and 2.58 respectively.

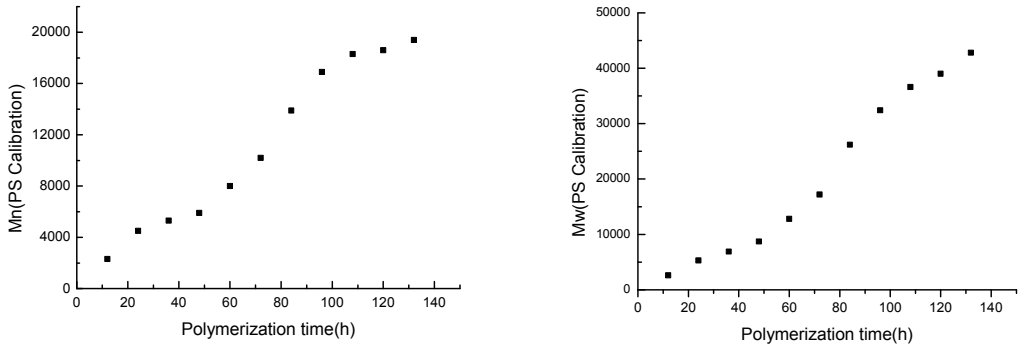
It was obvious that the polymerization rate was very slow at 125 ^oC. After 8 days (192 hours), the M_n was around ten thousand Da. The polymerization rate was of course very low because of the low reactivity of blocked isocyanate groups. Moreover, a (tin) catalyst, which is normally used to increase the reaction rate, was omitted to prevent (successfully) branching. But, too long reaction times could result in side reactions, such as oxidation, which is not acceptable.

3.3.2. Bulk polymerization of BLDI-OMe and PCL530

The polymerization was performed without any solvent at 125 ^{o}C. The polymerization was continued until 132h, while taking every 12h a sample. The caprolactam formed during the reaction evaporated because of the high temperature and vacuum conditions applied during the polymerization. As a result, a pure polymer was finally obtained. The resulting ^{1}H-NMR spectra were very similar to those in figure 3.3, while the caprolactam signals at 7.0 and 3.3 ppm were absent. The molecular weights increased in time, similarly as found with the solution polymerization (table 3.3 and figure 3.3), but now much faster.

Table 3.3 Molecular weights obtained by SEC of polyurethanes after various polymerization times, in bulk condition (T = 125 ^{o}C), using polystyrene standards

Time (h)	12	24	36	48	60	72	84	96	108	120	132
M_n (Da)	2,300	4,500	5,300	5,900	8,000	10,200	13,900	16,900	18,300	18,600	19,400
M_w (Da)	2,600	5,300	6,900	8,700	12,800	17,200	26,200	32,400	36,600	39,000	42,800
PDI	1.17	1.19	1.31	1.47	1.60	1.70	1.88	1.92	2.00	2.10	2.20



(a) **(b)**

Figure 3.3 The relationship between molecular weights and reaction time of the bulk polymerization of PCL and BLDI-OMe at 125^{o}C: (a) M_n vs time (b) M_w vs time.

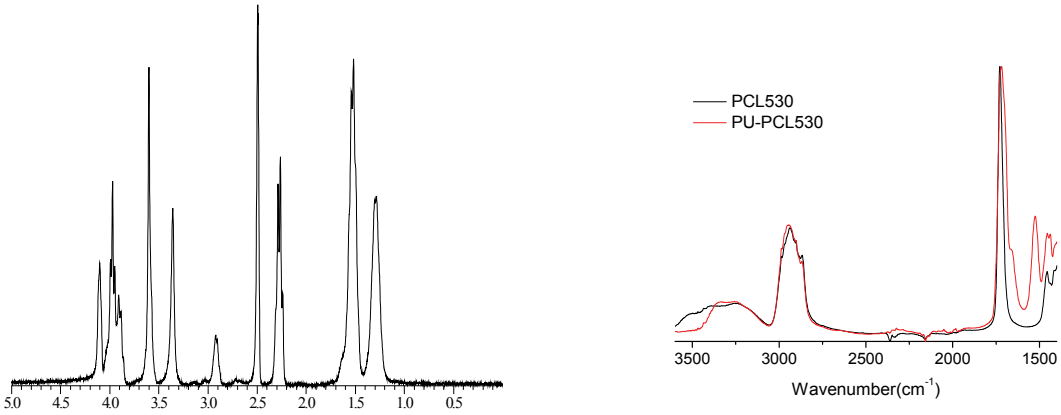
After 108 hours reaction, the M_n was already 18,000 Da in bulk conditions, while it was only 7,000 Da in solution. Notice that the PDI is still only 2.1, which is very close to 2.0, as it should be for polycondensations, without branching reactions. After a

polymerization time of about 100 hours the increase of the molecular weights slowed down, because of the high viscosity of system and of the low concentration of functional groups left. But, the molecular weights still increased slowly, but significantly. To reduce the length of the polymerization and to increase the molecular weights further, higher reaction temperature were investigated, although branching could take place.
In table 3.4 the results of the bulk polymerization (industry favored) at higher temperatures are compiled.

Table 3.4 The molecular weights determined by SEC of polyurethanes prepared from PCL and BLDI-OMe in bulk at various temperatures

T (^{o}C)	Time (h)	M_n (Da)	M_w (Da)	PDI
145	6	17,400	33,000	1.91
145	22	35,400	80,100	2.26
160	4	13,000	22,400	1.73
160	24	56,300	165,000	2.93
180	4	36,800	333,500	9.06

As shown in table 3.4, a number average molecular weight of 17,000 Da and PDI of 1.9 was obtained at 145 ^{o}C after 6 hours reaction, which is equal to reaction at 125 ^{o}C for 96 hours. Notice that the PDI is still 1.9, indicating that no branching took place. Polymerization time of 6 hours is convenient and very common, also in industrial processes. The number average molecular weight increased till 35,400 Da with PDI of 2.2 after 22 hours at 145^{o}C. The polydispersity of these polymers is still remarkably narrow, which means that the methyl ester group did not significantly react with the hydroxyl group of PCL530.
The ^{1}H-NMR results gave further evidence for the formation of only linear polymers. From figure 3.4(a), it can be seen that the sharp singlet signal of methyl ester COOC$\underline{H}_3$ (3.6 ppm) is still there and is even completely maintained. The methyl ester groups are amazingly stable even when heated to 160 ^{o}C in a bulk polymerization.



(a) (b)

Figure 3.4 Spectra of a polyurethane with a M_n of 35,400 Da and PDI of 2.26, obtained from a bulk polymerization at 145 °C for 22 hours, starting from BLDI-OMe and PCl530 (a): ^{1}H-NMR spectrum ($CDCl_3$); (b):ATR-FTIR spectra.

The conversion of PCL530 into PU was further supported by the ATR-FTIR analysis. From the ATR-FTIR measurement (Figure 3.4(b)), two distinctive vibration bands have been detected in the PU sample that was not present in the PCL530 spectrum: 1672cm^{-1} (shoulder of peak 1720cm^{-1}) and 1525cm^{-1}, originating from urethane carbonyl stretching vibration (amide I) and NH deformation vibration (amide II). The peak arranges from 3100cm^{-1} to 3450cm^{-1} and from 2814cm^{-1} to 3050cm^{-1} are related to the NH stretching vibration and CH_2, CH_3 stretching vibration respectively. The highest sharp peak at 1720cm^{-1} consists of ester carbonyl stretching vibration from both PCL530 ester and methylester side group of blocked diisocyanate. The absorption intensity is big because the urethane carbonyl stretching vibration peak is partly overlapped by the ester carbonyl peaks.[33]

When heated to 180 °C, branching occured, demonstrating that the methyl ester group reacted with the hydroxyl group of PCL530. This was evidenced by a broad polydispersity index of 9.06.

These results revealed an unexpected, but very interesting discovery that the bulk polymerization conditions of PCL and BLDI-OMe showed a high selective reactivity of

the hydroxyl groups of PCL for caprolactam blocked isocyanates and the pendant methyl ester. Only the caprolactam blocked isocyanate groups reacted with hydroxyl groups of PCL, whereas the ester group remained intact up to 160 ^{o}C. Nevertheless, BLDI-OMe behaved in bulk polymerizations as a trifunctional monomer above 180 ^{o}C, which could be used to synthesize polyurethane networks. Hence, the degree of polymerization and branching was tunable by varying the polymerization temperature and time.

3.3.3. Glass Transition temperature (T_g) of PUs depend on BLDI-OMe and PCL530

All our PU samples were transparent sticky wax like polymers without any indication that crystallization took place. The lack of crystallinity was considered as an important advantage to prevent inflammation reactions, when the polymer degrades in the body, due to remnant crystal moieties. Hence, it was expected that our PUs would not have detectable melting temperatures. Possible melting temperatures and glass transition temperatures (T_gs) were determined by DSC. The samples were heated from -60^{o}C to 90^{o}C, at heating rate of 10^{o}C/min. Table 3.5 give the glass transition temperatures (T_gs) of PUs with different molecular weights.

Table 3.5 Glass transition temperatures (T_g) of polyurethanes from PCL and BLDI-OMe, with various molecular weights which were prepared in bulk reaction.

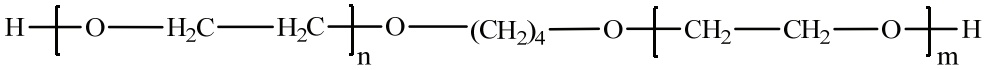
Sample(P#)	M_n (Da)	M_w (Da)	PDI	T_g (^{o}C)
1 PUOMe-PCL530	4,300	6,500	1.5	-32.8
2 PUOMe-PCL530	9,900	19,200	1.9	-28.1
3 PUOMe-PCL530	23,900	48,500	2.0	-26.0
4 PUOMe-PCL530	36,900	90,100	2.44	-35.4
5 PUOMe-PCL530	52,500	169,800	3.24	-26.3

None of the samples showed any indication of meting transitions. All the samples show low T_g values in a range of -33 ^{o}C to -26 ^{o}C, because of the flexible polyester spacer segment (PCL530). All PUs had the same soft segment (same chain mobility) which resulted in the similar T_gs. The T_g did not increase with increasing molecular weights of the PUs, and even not in branched polymers or in PU networks (chapter 4).

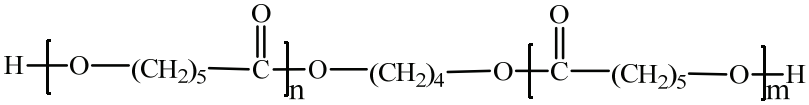
3.3.4. Bulk polymerization of BLDI-OMe and PEG600

PEG600 was selected as a representative example of polyether diols to synthesize PUs with the caprolactam blocked lysine diisocyanate methyl ester. As discussed before, we found that the bulk polymerization of a blocked diisocyanate with PCL530 was faster, as compared with solution polymerization in DMF. Accordingly, the polymerizations of PEG600 and BLDI-OMe were performed at 125^{o}C in bulk as well. However, after a polymerization time of 72h, an unexpected gelation took place, which was not found with PCL530. The hydroxyl groups of PEG have apparently a higher reactivity towards the ester group than those of PCL's (figure 3.5). One can speculate that hydroxyl groups of PEG are more acidic than the one of PCL. The hydroxyl group in PEG is two methylene groups apart from the electron withdrawing β-oxygen atom. The hydroxyl group in PCL is five methylene groups away from the ε-carbonyl group, resulting in a less acidic hydroxyl group.

The pKa value of hydroxyl functional compounds is a measure for the degree of dissociation of compound in a proton and an anion and is a measure of the acid strength. Numerous pKa prediction models have been developed, based on various approaches. M. Meloun and S. Bordovska[34] compared the predicted values obtained with four software packages (PALLAS, MARVIN, ACD/pKa DB, and SPARC) to experimental results of three sets of compounds. They concluded that the ACD/pKa DB model provided the most accurate predicted values, showing the best results in six different statistical characteristics. By using the online available ACD/pKa DB software we calculated that pKa values of PEG and PCL were 14.6 and 15.5 respectively. Although the differences were not large, it still meant that the hydroxyl group of PEG was more acidic than the one of PCL's, resulting apparently in more active species towards the methyl ester.



PEG



PCL

Figure 3.5 Structures of PEG and PCL

3.3.4. Conclusion

The poly(ester-urethanes) were successfully synthesized from our homemade caprolactam blocked lysine diisocyanate methyl ester and either polycaprolactone530 or PEG600.

In solution as well as in bulk a typical step-growth polymerization with PCL was observed. Molecular weights (M_n) up to 10,000 Da were obtained in the solution polymerization. As expected the bulk polymerization was much faster than the polymerization in DMF. In bulk molecular weights (M_n) up till 35,000 Da were obtained, with still a rather narrow molecular weight distribution of 2.26. Remarkably, the hydroxyl groups of PCL reacted in bulk nearly exclusively with the blocked isocyanate groups and not with the methylester group. Linear polyurethanes were obtained in the bulk polymerization, when the reaction temperature was below 160^{o}C. Bulk poly(ester-urethanes) networks can be obtained at temperatures above 180^{o}C. By varying the polycaprolactone molecular weights and the crosslink density the mechanical properties can be tailored.

Next to the polyester diol (PCL), a commonly used polyether diol, PEG, was heated with BLDI-OMe to make another series of non-crosslinked polyurethanes. In contrast to PCL, the hydroxyl groups of PEG600 reacted already at 125^{o}C (deblocking temperature of caprolactam blocked isocyanate group) with the pendant ester group, resulting directly in poly(ether-urethane) networks with useful mechanical properties (Chapter 4).

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Chapter 4

Polyurethane networks based on caprolactam blocked lysine diisocyanate

Abstract

In this chapter the synthesis of amorphous polyurethane networks is described, based on polydiols and on a homemade caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe). Poly(ε-caprolacton) and poly(ethyleneglycol) were selected as representative examples for the two classes of polyurethanes (PUs). The polyurethane networks were prepared in a single-stage process. After mixing the monomers, the curing was performed by heating the samples for 48 hours at 160^{o}C under vacuum. The PU network films, based on both PEG600 and PCL530, were flexible and totally transparent solids, which indicated that no crystalline phases were present. This was supported by DSC measurement. The polymer films swelled but did not dissolved in chloroform, demonstrating the crosslinked topology. The PU-PEG600 network was more hydrophilic than PU-PCL530 network, which resulted in a higher absorption of water. *In vitro* degradation test showed that the PU-PEG600 network degraded completely in 35 days, whereas PU-PCL530 lost about 3.5wt% of weight during this period. Mechanical measurements revealed that both polymers displayed rubber-like behavior. The lack of yield points in stress-stain curves demonstrated the amorphous character.

4.1. Introduction

Biodegradable polymers are receiving more and more attention due to their wide use in biomedical application.[1,2] They may act as temporary scaffolds to facilitate tissue regeneration or replacement and may also be used for temporary therapeutic purposes, eliminating the need for subsequent removal.[3,4] The majority of the biodegradable polymers developed in the last two decades were aimed at either drug delivery systems[2] or fracture fixation devices and are typically hard, rigid materials[5]. An important aspect of tissue engineering is the design of scaffolding materials which mimic the properties of extracellular matrices. Moreover, due to the temporarily character in this application, a tunable degradation behavior is mandatory. In this respect, the physical-, chemical- and biological properties and the biodegradation behavior of the materials are decisive for their suitability.

A variety of hydrolytically degradable polymers have been developed for tissue engineering scaffold applications. However, the majority of these polymers consisted of high molecular weight linear aliphatic (co)polyester. These materials often possess mechanical properties best suited for hard tissue engineering because of their relatively higher glass transition temperature and high modulus.[6-10] For engineering of soft tissues, elastic scaffolds are desirable since they are amenable to mechanical conditioning regimens that might be desirable during tissue development.[11] For example, the elastic moduli are ranging from approximately 400 MPa for poly(ε-caprolacton) to 3000 to 3500 MPa for poly(glycolide) and poly(lactide), while soft tissue, such as myocardium, have elastic moduli below 1 MPa.[12-14]

Polyurethane elastomers have been used as biomaterials for several decades in the fabrication of medical implants such as cardiac pace makers and vascular grafts because of their unique physical properties and relatively good biocompatibility as well as their biodegradability.[15,16] In order to achieve good mechanical properties, the molar weight of linear polyurethanes has to be relatively high, to have a sufficient quantity of a crystalline

hard phase to display an appropriate physical crosslinking. However, crystalline residues that stay behind after degradation caused inflammatory reactions *in vivo*.[17,18]

It has been reported that polyurethanes, based on lysine diisocyanate, degrade by hydrolysis of urethane linkages yielding lysine,[19,20] while others have reported that urethane and urea linkages are enzymatically degraded.[21,22] Hence, polyurethanes and polyurea based on lysine are apparently (bio) degradable, and are therefore suitable for temporarily scaffolds.

As described in Chapter 3, transparent non-crystalline linear polyurethanes were successfully synthesized from our homemade caprolactam blocked lysine diisocyanate methyl ester and PCL530. Interestingly, it was noticed that poly(ether-urethane) formed already networks at 125^{o}C (without catalysts), which is the minimum temperature at which deblocking of caprolactam blocked isocyanate groups starts. In contrast, poly(ester-urethane) needed at least a temperature of 200 ^{o}C to form crosslinked materials, which may result in side reactions, such as transesterifications. However, we have showed earlier (chapter 2) that the methyl ester group of caprolactam blocked lysine diisocyanates reacted with 1-octanol at 125^{o}C in the presence of a tin catalyst. This offered the possibility to synthesize also poly(ester-urethane) networks at lower temperature.

In this chapter, the synthesis is reported of non-crystalline polyurethane networks that were obtained from caprolactam blocked lysine diisocyanate methyl ester (prepared according to the method as described in Chapter 2) and polycaprolactone or poly(ethylene glycol) diols. The synthesis, characterizations and properties of the PUs are described in detail.

4.2. Materials and Methods

4.2.1. Materials

CBC (carbonyl biscaprolactam) was kindly obtained from DSM Innovation Center, ALLINCO®, (> 99 % pure according to HPLC) and used without purification.

L-Lysine monohydrochloride, thionylchloride, calciumchloride dihydrate, anhydrous magnesium sulfate, tin(II)2-ethylhexanoate, poly(ethyleneglycol)600 (PEG600), poly(ethyleneglycol)1000 (PEG1000), and poly(ε-caprolacton)530 (PCL530) were purchased from Sigma-Aldrich.

Poly (ε-caprolacton) 1250 (PCL1250) was purchased from Polysciences Inc.

Ethyl acetate and propylene glycol, were purchased from Acros Organics.

Methanol, acetone, chloroform, triethylamine (TEA), n-hexane, hydrochloric acid fuming 37% and tetrahydrofuran (THF) were purchased from Lab-Scan Analytical Science.

Phosphate buffered saline (PBS, pH=7.4) was obtained from Apotheek (Pharmacy) UMCG (University Medical Center of Groningen, the Netherlands). All purchased compounds were used as obtained.

Caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe) was synthesized according to the method which have been described in chapter 2.

4.2.2 Synthesis of polymer networks

Polyurethane networks were prepared by using a single-stage process. A mixture of appropriate amounts of BLDI-OMe and a polydiol (in a molar ratio of 1:1.1) were heated at 100^{o}C to reduce the viscosity. Only when PCL was used as polydiol 2.5wt% of tin(II)2-ethylhexanoate was added as a catalyst. The mixture was subsequently poured into a preheated open mold (Figure 1) and cured for 48 hours at 160^{o}C under vacuum. The size of the strip shaped PU networks was 40x3.0x0.8mm

Figure 4.1. The teflon mold for preparing polyurethane networks. The dimension of the strip space of mold was 40x3.0x0.8mm.

4.2.3 Characterizations of polymer networks

Differential Scanning Calorimetry (DSC) measurements were performed on a Perkin Elmer DSC 7 instrument. About 10 mg of the samples were heated from -80 ^{o}C to +90^{o}C with a rate of 10^{o}C/min.

ATR-FTIR measurements were performed on a broker IFS88 spectrometer equipped with a Golden Gate (Graseby specac) single reflection ATR accessory. Spectra resolution was 4 cm^{-1} and 32 scans were taken per spectrum.

Gel contents (in wt%) were determined by extraction of the networks with chloroform. The extracted networks were first carefully air-dried and then dried in vacuum at 40 ^{o}C until a constant weight was reached. The gel content (%) was defined as: [W_d/W_o]*100%, where W_d represents the weight of the sample after drying and W_o represents the weight before immersing in chloroform.

Swelling measurements were carried out on non-extracted polymer networks in chloroform at room temperature. After immersing the films for 24h in chloroform they were dried with a tissue and weighted immediately. The degree of swelling was

calculated from the weight increase after swelling using the following equation: the degree of swelling (%) = $[(W_s - W_o) / W_o]*100\%$, where W_s represents the weight of the sample swollen in chloroform and W_o represents the weight before immersing in chloroform.

The equilibrium water uptake of non-extracted thin film was determined after conditioning at 37°C in distilled water for three days.[23] Water uptake was defined as the mass increase of the thin film: water uptake (wt %) = $[(W_w - W_o)/W_o]*100\%$, where W_w represents the weight of the wet sample after immersing and W_o represents the weight before immersing in water.

The contact angle measurements were carried out at room temperature by using a custom built microscope-goniometer system. A 1.00 μL drop of the deionized water was placed on a prepared PU network film using a Hamilton micro-syringe and the contact angle was measured after 60 s. The measurements involve the fitting of a drop picture with home-built software. Four measurements were carried out in different regions on the surface.

Mechanical properties were determined at room temperature using an Intron (5565) tensile tester equipped with a 100N load-cell at a cross-head speed of 10 mm/min. Specimens (40x3.0x0.8mm) were prepared in the open mold (Figure 1).

In vitro degradations were performed in phosphate buffered saline (PBS, pH=7.4). Non-extracted, strip like specimens (10x3.0x0.8mm, cut from origin samples) (n=3/time point) were placed in vials containing 20 mL of PBS at 37°C in the dark oven. PBS was changed after each analysis point. At predetermined times, the mass was determined after drying the specimens to constant weight in vacuum at 40°C. The weight loss was defined as follows: weight loss (%) = $[(W_o - W_t)/W_o]*100\%$, where W_o represents the weight of the dry sample before degradation and W_t represents the weight of the dry sample after degradation at various time intervals.

4.3. Results and discussion

4.3.1. Synthesis

One of the most widely used techniques to obtain polyurethanes is accomplished in a very efficient and fast way by mixing, in a one-step procedure, polyols, isocyanates and chain extenders .[23]

The polyurethane networks described in this chapter were prepared from the novel caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe, synthesized as described in chapter 2) and various polyols, by using a one-step processing technology. As the aim of this project was to make amorphous PUs, low molecular polydiols, such as PEG600, PEG1000, PCL530 and PCL1250, were used as soft segments to suppress crystallization. As described earlier (chapter 3), it was not necessary to use catalysts for synthesis of poly(ether-urethane) networks, but it was indispensable for poly(ester-urethane). Commonly used catalysts, such as amines or organometallic compounds, may be toxic. Among them, tin(II)2-ethylhexanoate (commonly referred as stannous octoate) is a good choice, as this catalyst is accepted by the Food and Drug Administration (FDA) in coatings in food contact applications .[24]

By varying the molar ratio of raw materials, the reaction temperatures and the polymerization times, PUs with different properties can be obtained, depending on the intended applications. In this study, the molar ratio between polydiols and BLDI-OMe was fixed to 1.1:1. 2.5wt% tin(II)2-ethylhexanoate used when PCL was the polyol of choice. The reactants were stirred at 100^{o}C for 5min to form a homogenous mixture and were then cured in a mold for 48 hours at 160^{o}C under vacuum. Under the applied conditions, the blocked diisocyanate as well as the methyl ester group of BLDI-OMe were able to react with hydroxyl groups, forming PU networks. The liberated caprolactam and methanol evaporated during the curing process, due to the high temperature and vacuum condition.

The linear polyurethanes, based on BLDI-OMe and short soft segments polydiols, were sticky at room temperature and were free flowing viscous liquids when heated. In contrast, the crosslinked polyurethanes were not sticky at all and could not flow any more, even not at 220^{o}C. The PU network films, based on PEG600 as well as on PCL530, were flexible and totally transparent solids, which indicated that no crystalline phases were formed. The films based on the higher molecular polydiols, PEG1000 and PCL1250,

appear to be stiff and had a white appearance, which indicated that crystalline phases were formed.

4.3.2. DSC measurements

The absence of crystallinity was supported by DSC measurement. For that, the samples were heated from -80°C to +90°C, at heating rate of 10°C/min. Figure 4.2 shows the second heating thermogram of various polyurethanes. The thermal transitions are listed in Table 4.1. For PU-PEG600 and PU-PCL530 networks, there was no sign of crystallization (no T_m) visible, but they were clearly visible in PU-PEG1000 and PU-PCL1250 samples. Obviously, longer soft segments allowed polymer chains to align, resulting in crystalline phases in these samples. The crystallinity of PU-PCL1250 was more pronounced than of PU-PEG1000. As the aim of this study was to prepare amorphous polyurethanes, we focused on non-crystalline PU-PEG600 and PU-PCL530 networks for further investigations.

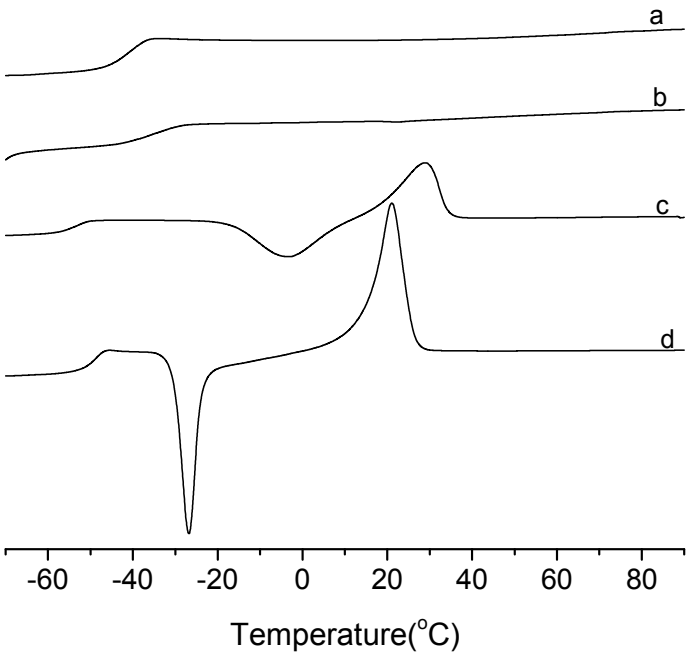


Figure 4.2 DSC thermograms (10°C/min) of PU networks:(a) PU-PEG600, (b) PU-PCL530, (c) PU-PEG1000, (d) PU-PCL1250

Table 4.1 Glass transition temperature (T_g) and melting temperatures (T_m) of PU networks

Samples	T_g (^oC)	T_m (^oC)
PU-PEG600	-40.5	none
PU-PEG1000	-48.5	21.1
PU-PCL530	-30.7	none
PU-PCL1250	-53.8	28.4

4.3.3. ATR-FTIR analysis

The PU-PEG600 and PU-PCL530 networks were further investigated by the ATR-FTIR analysis (Figure 4.3). The peaks ranging from 3200 cm^{-1} to 3450 cm^{-1} and from 2814 cm^{-1} to 3000 cm^{-1} were related to the N-H stretching vibrations and C-H (CH, CH_2 and CH_3) stretching vibration respectively. In PU-PCL530 spectrum, the band at 1710 cm^{-1} was due to the urethane and the ester carbonyl (from PCL530 and methyl ester of BLDI-OMe) stretching vibration. Consequently, the absorption intensity of that peak of the PU-PCL530 network was bigger than that of PU-PEG600 network. The strong peak at 1100 cm^{-1} of PU-PEG600 was attributed to the C-O-C stretching from polyether PEG600 segment.

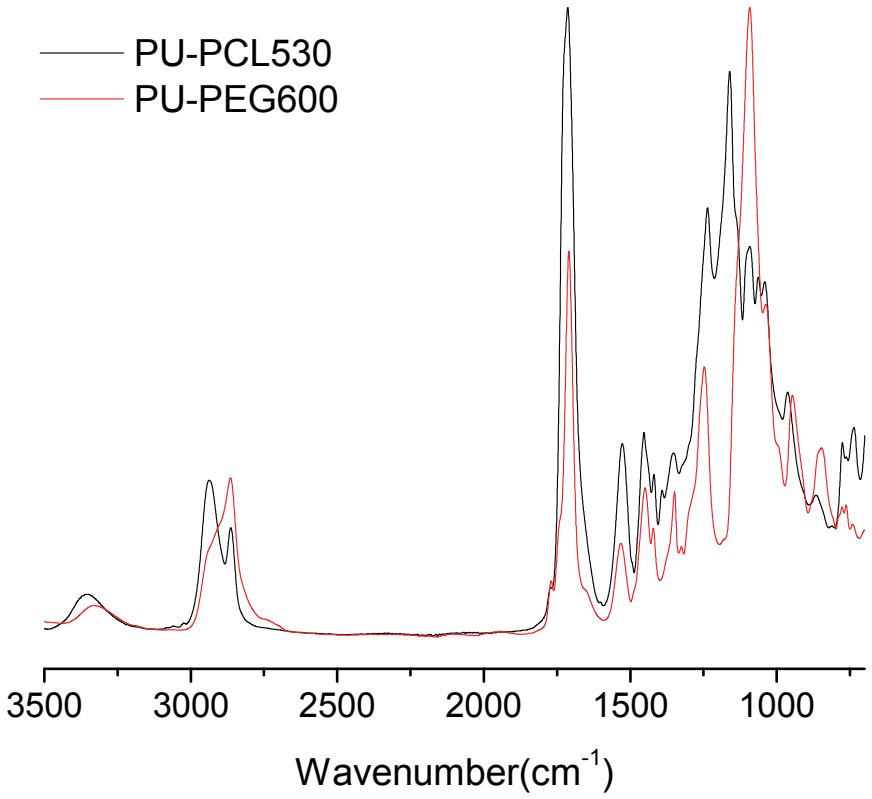


Figure 4.3 FTIR spectra of PU-PCL530 and PU-PEG600 networks

4.3.4. Swelling tests

Next, the PU networks were characterized by performing swelling tests using chloroform as swelling medium. Water uptake and water contact angles were also measured to determine the polyurethane bulk and surface polarity. The gel contents, degrees of swelling, water uptake and water contact angle of the networks are summarized in Table 4.2.

Table 4.2 The swelling, water uptake and contact angles of PU-PEG600 and PU-PCL530 networks

Samples	Degree of swelling in chloroform (%)	Gel content in chloroform (%)	Water uptake (%)	Water contact angle (^{o})
PU-PEG600	730	94.9	189	68.5± 3
PU-PCL530	1100	85.7	102	88.5± 3

In contrast to the linear polymers, all the crosslinked samples did not dissolve in chloroform,, but swelling occurred. The weight increase in chloroform was seven fold and eleven fold for PU-PEG600 and PU-PCL530 networks, respectively. The polymer with the highest gel content (94.9) had the lowest chloroform swelling degree. However, we cannot conclude here that there is relation between the lower gel content and the amount absorbed chloroform, because the difference in polarity of the polyester and polyether diols is much more important. Water uptake was measured to determine hydrophilicity of the polymer networks. It was expected that this would have a substantial impact on hydrolytic degradation rate. The PU-PEG600 network can absorb more water (89wt%) than PU-PCL530 networks (2wt%), because of the hydrophilic nature of PEG600.

The water contact angle is a measure for the surface polarity. The PU-PEG600 network (68.5^o) was, as expected, more hydrophilic than PU-PCL530 (88.5^o).

4.3.5. Mechanical properties

The mechanical properties of polyurethane networks are shown in Figure 4.4 and in Table 4.3. The properties showed a rubber-like behavior. The lack of yield points in the whole stress-strain curves of PU-PEG600 and PU-PCL530 demonstrated that the amorphous character retained even under stress, (Figure 4.4). No signs of strain-hardening were observed. As shown in Table 4.3, the rubber like behavior of PU-PEG600 and PU-PCL530 was also apparent from the elastic modulus, elongation at break and

stress at break, which were 1.64MPa and 0.65MPa, 101% and 164%, 0.88MPa and 0.57MPa, respectively. In this case, the PU-PEG600 had higher modulus and stress at break but lower elasticity than PU-PCL530. By varying the synthesis parameters, it is possible to obtain different mechanical properties, which can be utilized to fulfill the requirements of numerous biomedical applications. The PU-PEG600 and PU-PCL530 networks in this study were designed to be in the range of scaffolds for soft tissue.[12-14]

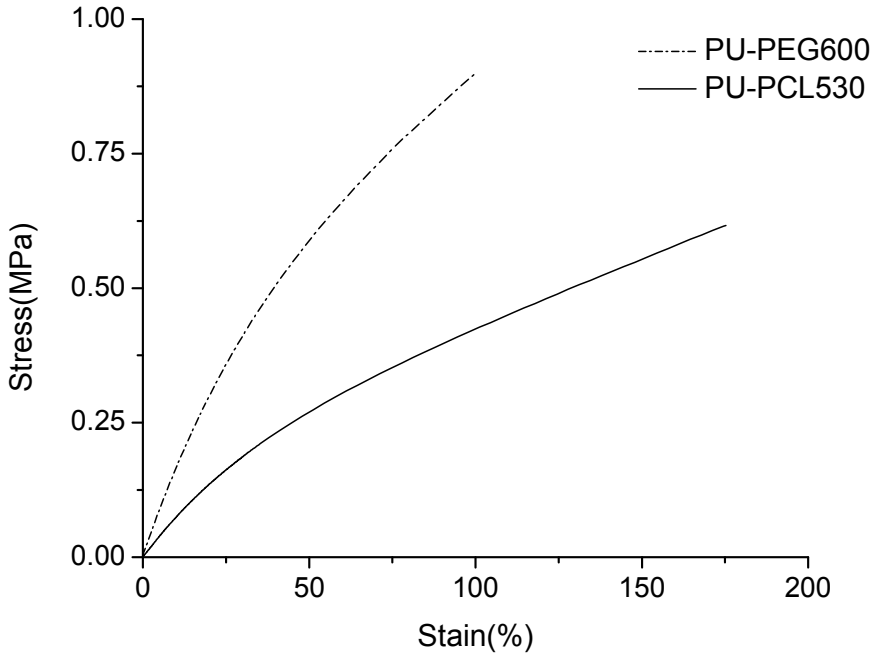


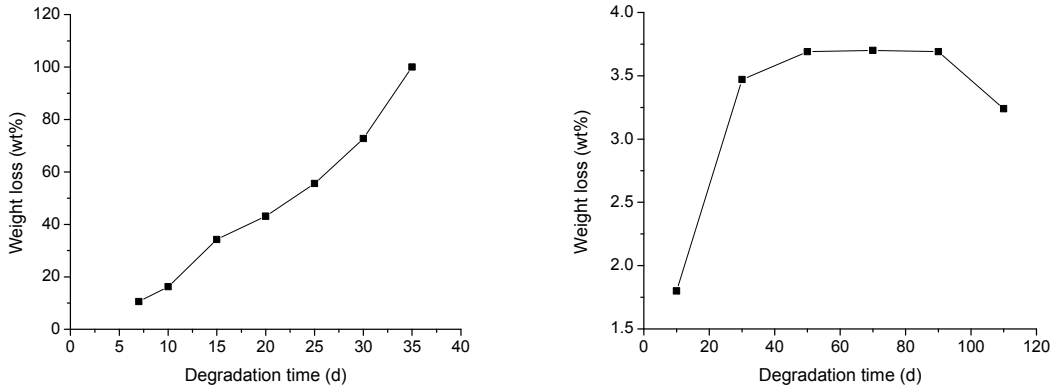
Figure 4.4 Stress and strain curves of PU-PEG600 and PU-PCL530 networks

Table 4.3 Mechanical properties of PU-PEG600 and PU-PCL530 networks

Samples	Modulus (MPa)	Elongation at break (%)	Stress at break (MPa)
PU-PEG600	1.64±0.2	101±10	0.88±0.04
PU-PCL530	0.65±0.1	164±15	0.57±0.04

4.3.6. Hydrolytic degradation

The *in vitro* degradation experiments of the polyurethane networks were carried out by immersing samples in PBS (pH 7.4) at 37°C, while following the weight losses in time. The results are plotted in Figure 4.5. Please note the difference is scale of the Y-axis of both compounds. The PU-PCL530 network showed a very low mass loss, of about 3.5% after 110 days. However, this is probably due to extractables, and not to hydrolysis. This might explain why the weight loss leveled off after the first few days. A slow degradation was expected from PCL-based polyurethanes, which are commonly used in long lasting biomedical applications.[25,26] It is generally believed that *in vitro* and *in vivo* degradation of polyester urethanes proceeds via autocatalytic hydrolytic chain scission of the ester bond of the polyester soft segment.[27] In contrast, the PU-PEG600 network exhibited progressive mass loss over 35 days ranging finally up to 100%. The higher hydrophilicity of the PU-PEG networks (corresponding to water uptake results) resulted in much faster degradation rate.[28,29] The PU-PEG600 network could be used as short term materials. Depending on the applications, the polyester and polyether diols co-polyurethanes could be prepared to form soft segments with intermediate degradation times.



(a) (b)

Figure 4.5 In vitro degradation (PBS, pH 7.4, 37°C) of polyurethane networks: (a) PU-PEG600, (b) PU-PCL530.

4.4. Conclusion

The polyurethane networks were successfully synthesized based on polydiols and our homemade caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe) by using a one-step processing technology. PEG600, PEG1000, PCL530 and PCL1250 were used as polydiols to study the synthesis of non-crystalline PU networks. Only the PU network films based on PEG600 and PCL530 gave non-crystalline materials, as evidenced by their transparency, by DSC measurement and by the lack of yield points in stress-strain curves. These networks swelled, but did not dissolve in chloroform, demonstrating the crosslinked structure. In the mechanical tests, both networks showed rubber-like behavior.

The PU-PEG600 network was more hydrophilic than PU-PCL530 network, and could, as a consequence, absorb more water. In *in vitro* degradation tests, the PU-PEG600 network degraded much fast than the PU-PCL530 networks. PU-PEG600 needed only 35 days to degrade 100%, while the PU-PCL530 network lost about 3.5% of weight (extractables) in the same period. The PU-PEG600 and PU-PCL530 networks in this study are potentially suited for scaffolds for soft tissue.

Reference

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Chapter 5

Polyurethanes provided with pendant covalently grafted GRGDG peptide moieties

Abstract

Poly(ester-urethanes), comprising pendant activated N-hydroxysuccinimide (NHS) esters, were prepared from the NHS-activated ester of caprolactam blocked lysine diisocyanate and polycaprolactone. The NHS-activated ester offered an enabled technology to prepare bio-active polyurethanes. First, hexyl amine was used, as model compound, to substitute the NHS-group, demonstrating the ability of the concept. Along the same line, an amino-functional, cell-adhesion promoting peptide, GRGDG, was coupled onto polyurethane backbones. NMR, IR and elemental analysis proved the successful tethering of both, hexyl amine and the GRGDG peptide, on the polyurethane backbone. XPS and water contact angle measurement of spin-coated polyurethanes on glass slides confirmed the presence of the grafted peptides, showing a strong change of the surface property of polymer. The water contact angle went down from about 85^{o} to 20^{o}, demonstrating the highly hydrophilic character due to the GRGDG moiety. *In vitro* cell adhesion experiments with human dermal fibroblast (HDF) cells showed that, with and without the presence of serum in culture medium, the GRGDG-grafted polyurethanes impressively enhanced cell attachment compared with the starting polyurethanes. In serum-free circumstance, the GRGDG-grafted polyurethane showed even a better cell survival and spreading than on tissue culture polystyrene (TCPS).

5.1. Introduction

For decades a large number of synthetic polymeric materials with a variety of properties are available for biomedical applications,[1] like in prostheses, implants and tissue engineering matrices. Most of the materials have sufficient mechanical properties, as well as desired stability towards degradation or display a controlled degradation behavior.[1] They are biocompatible and produce during degradation only non-toxic substances. An important problem still is the inadequate interaction between the polymers and human cells, leading *in vivo* to foreign body reactions, such as inflammation, aseptic loosening, local tissue waste, and implant encapsulation as well as thrombosis and embolization.[2] Approaches to improve biomaterials include reduction of unspecific protein adsorption, known as non-fouling properties, enhancement of specific protein adsorption and material modification by immobilization of cell recognition motives to obtain controlled interaction between cells and synthetic substrates. [3-12]

Since RGD peptides (R: arginine; G: glycine; D: aspartic acid) have been found to promote cell adhesion in 1984 (Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule, Nature 309 (1984) 30), numerous materials have been RGD-functionalized in academic studies for medical applications. The RGD sequence is also proved by far to be the most effective and most often employed peptide sequence for stimulated cell adhesion on synthetic surfaces. The RGD tripeptide sequence has been found in vitronectin, fibronectin, von Willebrand factor, fibrinogen, and collagens. This tripeptide has been shown to play a crucial role in mediating cell attachment and subsequent spreading.[13,14] When immobilized onto a surface, RGD-containing synthetic peptides promote cell attachment in a manner similar to that of fibronectin, whereas in solution the same peptides inhibit the attachment of cells to surfaces coated with fibronectin or with the peptides,[13] due to the blocking of the integrins. It has been shown that some cell receptors (i.e., integrins) bind to the RGD sequence of a single protein only, whereas other receptors recognize RGD sequences in various proteins.[15]

The process of integrin mediated cell adhesion comprises a cascade of four different partly overlapping events[5]: cell attachment, cell spreading, organization of actin

cytoskeleton, and formation of focal adhesions. Firstly, in the initial attachment step the cell contacts the surface and some ligand binding occurs that allows the cell to withstand gentle shear forces. Secondly, the cell body begins to flatten and its plasma membrane spreads over the substratum. Thirdly, this leads to actin organization into microfilament bundles, referred to as stress fibers. In the fourth step the formation of focal adhesions occurs, which link the ECM (extracellular matrix) to molecules of the actin cytoskeleton.

Progress in understanding the interactions of RGD-containing peptides and cell receptors has stimulated a great deal of interest in the development of novel biomaterials that may improve long-term cell attachment and growth. Synthetic RGD-containing peptides immobilized on poly(tetrafluoroethylene), poly(ethy1eneterephthalate),[16,17] poly(viny1 alcohol),[18] polyacrylamide,[19] and poly(carbonate urethane)[20] surfaces have been shown to support cell attachment and spreading.

Polyurethanes (PUs) are widely used as cardiovascular biomaterials due to their good blood compatibility,[21-23] biodegradability and mechanical properties. However, cellular adhesion on PU materials is relatively poor. Various methods have been developed to promote the cell adhesion property. These include bulk modification of the chemical structure of PU,[24,25] surface modification such as ion beam irradiation[26] or plasma treatment[27], and coating with or grafting of adhesive proteins[28-30].

Previously, we successfully synthesized poly(ester-urethanes) (linear and networks) starting from our homemade caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe) and polycaprolacton530 (chapter 3 and 4). To utilize the methylester side group of BLDI-OMe, for the incorporation of RGD groups, the methylester had to be hydrolyzed. However, hydrolyzing the methylester without affecting the ester linkages of the polyester diols in the polymer backbone is impossible. For this, we took the advantage of the stability of blocked diisocyanate (BLDI-OMe). As we showed earlier (chapter 2), the methylester could be hydrolyzed under well-defined conditions without affecting the blocked isocyanate group.

Here we studied if the pendant acid group, that offered the possibility to make activated esters (chapter 2), could be used as a possible route to introduce RGD-peptide side-groups. If successful, the next challenge would be to demonstrate that these immobilized

peptides preserved their ability to improve the cell adhesion property of our polyurethanes.

5.2. Materials and Methods

5.2.1. Materials

CBC (carbonyl biscaprolactam) was kindly obtained from DSM Innovation Center, ALLINCO®, (> 99 % pure according to HPLC) and used without purification.

L-Lysine monohydrochloride, thionylchloride, calcium chloride dihydrate, hexyl amine, dibutyltin dilaurate (DBTDL, 95 %), 1-octanol, anhydrous magnesium sulfate, dicyclohexyl-carbodiimide (DCC), N-hydroxysuccinimide (NHS), α-naphthol, urea, poly(ε-caprolacton)530 (PCL530), chloroform-d ($CDCl_3$-D, 99.8 atom % D) and dimethyl sulfoxide-d_6 (DMSO-d_6, 99.5 atom % D) were purchased from Sigma-Aldrich.

Ethyl acetate, potassium hydroxide and anhydrous *N,N*-dimethylformamide (DMF), were purchased from Acros Organics.

Methanol, acetone, ethanol, chloroform, triethylamine (TEA), n-hexane, hydrochloric acid fuming 37%, tetrahydrofuran (THF), dichloromethane (DCM), were purchased from Lab-Scan Analytical Science.

Phosphate buffered saline (PBS, pH=7.4) was obtained from Apotheek UMCG (University Medical Center of Groningen, the Netherlands). All compounds and solvents were used as obtained, unless stated otherwise.

GRGDG was kindly obtained from by Dr. D. Löwik from the Radboud University of Nijmegen (the Netherlands).

Caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe) and BLDI-NHS were synthesized according to the methods as described previously (chapter 2).

The linear PCL530 based polyurethanes were synthesized according to the method as described in chapter 3.

5.2.2. Analysis

Column chromatography was performed on silica gel (Silica-P flash silica gel from Silicycle, size 40-63 μm). TLC was performed on silica gel 60/Kieselguhr F254. Components were visualized by UV and staining with a solution of a mixture of $KMnO_4$ (5g) and K_2CO_3 (20 g) in H_2O (500 mL).

Size exclusion chromatography (SEC) measurements were performed in dimethylformamide (DMF) with 0.01M LiBr on a Viscotek GPC max equipped with model 302 TDA detectors, using 2 columns (Pl-gel 5μ 30 cm mixed-C from Polymer Laboratories). The data analysis was done using conventional calibration with polystyrene standards accompanied by in-house software.

^{1}H-NMR was recorded on a Varian AMX400 using $CDCl_3$ or DMSO-d_6 as solvent.

Element analysis (EA) was performed on a HEKAtech Gmbh Euro-EA CHNanalyzer.

ATR-FTIR was done using a Broker IFS88 spectrometer equipped with a Golden Gate (Graseby specac) single reflection ATR accessory. Spectra resolution was 4 cm^{-1} and 32 scans were taken per spectrum.

Sakakgushi assay[20] was used for the detection of arginine in the samples. 0.5 mL of α-naphthol (0.01wt% in 95wt% ethanol +5wt% urea) and three pellets of NaOH were added to a glass tube containing approximately 5 mg of sample. After 5 min, 0.5 mL bromine solution (2wt% in 100 ml of 8wt% NaOH) was added to the tube. An orange-red solution appears in the tube as an indication of the presence of arginine.

Differential Scanning Calorimetry (DSC) measurements were performed on a Perkin Elmer DSC 7 instrument. The samples, with masses varying between 7-10 mg, were heated from -60 ^oC to +90 ^oC with a rate of 10 ^oC / min.

Surface property

Surface property characterization was performed on polymer-coated glass slides (1x1cm^2) and silica wafers (1x1cm^2). The glass slides and silica wafers were cleaned with chromic acid, washed with deionized water and dried. Subsequently, a 5% (w/v) polymer solution

in DMF was spin-coated (2,000rpm) on the substrates. The polymer-coated samples were dried under vacuum for 24h at 40^{o}C before characterization

The water contact angle measurements were carried out at room temperature by using a custom built microscope-goniometer system. A 1.00 μL drop of the deionized water was placed on a prepared polymer-coated glass slide using a Hamilton micro-syringe and the contact angle was measured after 30s. The measurements involve fitting of a droplet picture with home-built software. Three measurements were carried out in different regions on the surface.

The XPS (X-ray photoelectron spectroscopy) measurements were performed with a monochromatic small-spot X-ray Photoelectron Spectrometer System (Thermo Scientific K-Alpha) with an Al Kα monochromator X-ray Source (1486.6eV). The base pressure in the spectrometer during the measurement was 10^{-8} mbar.

Cell adhesion

Human dermal fibroblast cells (HDF; Sciencell, USA) were generously provided by Dr. Marco C. Harmsen (University Medical Center of Groningen, the Netherlands). Cells were harvested for the experiments by incubating them in accutase (enzyme cell detachment medium) for 3 min at 37°C. Half number of the cells was collected by centrifugation and re-suspended in EMEM (Eagle's minimum essential medium), additional with L-glutamine (2mM), sodium pyruvate (1mM)) and 10wt% bovine fetal calf serum (FCS). The other cells were re-suspended in same medium without FCS. Polymer-coated glass slides were placed in 24-well tissue culture plates (Corning) and sterilized for 15 min under ultraviolet light. Next, 1x10^{5}cells/mL were seeded on each polymer substrate and allowed to attach for a desired time. The wells were subsequently rinsed with PBS to remove non-adherent cells, and the adherent cells were fixed with a solution of 2wt% paraformaldehyde for 10min. Cells were stained with 0.5wt% DAPI (4',6-diamidino-2-phenylindole) in PBS for 1h. The cell number was determined by fluorescence microscopy utilizing a Leica DC 300F microscopy (Wetzlar, Germany). Adherent cells were counted in twelve or thirteen randomly chosen areas in the central

and peripheral regions of each polymer substrate. The morphology of attached cells was examined using microscope with bright field mode.

5.2.3. Synthesis of N-hydroxysuccinimide functional polyurethanes (PU-NHS)

A 100 mL glass flask was fitted with a nitrogen inlet and an outlet to a vacuum pump. After the flask was three times evacuated and refilled with nitrogen, to remove all oxygen, dry N-hydroxysuccinimide activated ester of caprolactam blocked lysine diisocyanate (BLDI-NHS) was added in a molar ratio of 1.025 with respect to PCL530. BLDI-NHS was synthesized according to the method as described previously (chapter 2). The mixture was subsequently heated up to 130, 135, 140 and 145^{o}C, respectively, while stirring under vacuum for 12 hours, and taking meanwhile samples.

5.2.4. Modification of PU-NHS with hexyl amine

PU-NHS was dissolved in 100 mL methylene chloride. A threefold excess of hexyl amine was added. The reaction mixture was stirred at room temperature overnight. After removal of precipitated NHS-amine salt by filtration, the solution was washed with a 5wt% $NaHCO_3$ aqueous solution and with brine (saturated NaCl aqueous solution). The resultant solution was dried over anhydrous $MgSO_4$. After removal of $MgSO_4$ by filtration the polymer solutions were poured into diethyl ether. The precipitate was separated by centrifugation and after the supernatant was removed the polymers were dried in the vacuum oven in 50 ^{o}C for 48 h.

5.2.5. Modification of PU-NHS with GRGDG

The GRGDR peptide and triethylamine (TEA) were added to a solution of PU-NHS (10% w/v) in DMF. The coupling reaction was performed at 50^{o}C under dry nitrogen during 48 hours. The peptide and TEA was added in a slight excess with respect to stoichiometric amounts of NHS groups of the polymer. After removal of precipitated NHS-amine salt by filtration, the peptide grafted polymer (PU-GRGDG) was precipitated

in deionized water. The polymer was collected by centrifugation and, after the supernatant was removed by decantation. The polymer was subsequently dissolved in DMF and further purified by pouring this solution into diethyl ether. The product was separated by centrifugation and after the supernatant was removed the polymers were dried in the vacuum oven in 50^{o}C for 48 h.

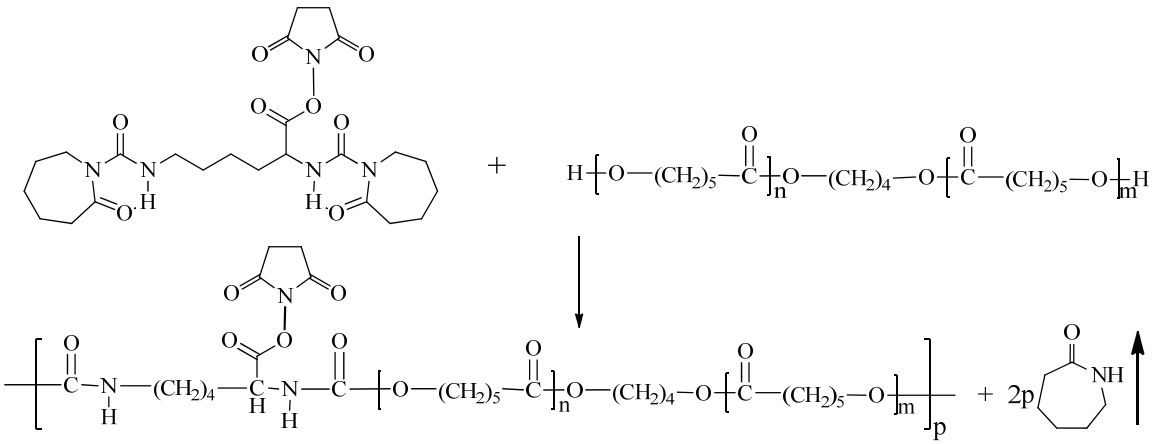
5.3. Results and discussion

5.3.1. Synthesis of PU-NHS, PU-HA and PU-GRGDG

Poly(ester-urethanes) are the workhorses of biomedical polyurethanes. They offer excellent mechanical properties and a can be tuned to give controlled hydrolytic degradation rates. As described in Chapter 3, linear poly(ester-urethanes) were successfully synthesized by our homemade caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe) and polycaprolactone 530. When no catalyst was used the pendant ester group of BLDI-OMe remained during the polymerization totally unaffected, which offered a very important tool for modification of polymers. However, to enable derivatization reactions, hydrolysis of the pendant ester group had to be done without cleaving the ester linkages of the polyester diols in the polyurethane backbone, which is difficult, maybe even impossible.

A successful methodology to hydrolyze the ester group of the caprolactam blocked lysine diisocyanate methyl ester was found, without destroying the blocked isocyanate function, which enabled the preparation of functionalized caprolactam blocked lysine diisocyanate. The esterification of the acid group with N-hydroxysuccinimide (NHS), yielding BLDI-NHS activated ester (chapter 2), offered a possible route to decorate poly(ester urethanes) with various functional groups. This could be done either in the monomer, before the polycondensation, or afterwards in the polymers, after the polycondensation. In the latter case the NHS activated ester had to survive the polymerization conditions. This was investigated first, as it would offer a convenient route to prepare functional PUs. The synthesis of BLDI-NHS has been reported previously in detail (Chapter 2). PCL530 was used here in this chapter as a representative example for polyesters. The reaction scheme

is depicted in Scheme 5.1. The polymerization was performed in bulk (Chapter 3) at temperature of 125°C and higher, and during various residence times. The only side product would be caprolactam, provided that NHS was not substituted. Caprolactam formed during the reaction, evaporated because of the high temperatures and vacuum conditions. As a result, a pure polymer could finally be obtained. The polymers dissolved well in DMF, proving that no crosslinking had taken place. Although, the NHS-ester is much more reactive than the methyl ester, it still survived the polymerization conditions, at least to a substantial extent. Since the polymers dissolved well in DMF the SEC analyses were therefore performed in the same solvent. Table 5.1 gives the molecular weights of PU-NHS based on BLDI-NHS and PCL530, which were obtained by varying the polymerization temperature and time.



Scheme 5.1 Synthesis of PU-NHS from blocked lysine diisocyanate (BLDI-NHS) and poly(ε-caprolacton) (PCL), polymerized without catalyst.

From Table 5.1 it can be seen that the PDIs of polymers with number average molecular weights up till about 8,000 Da were still quite narrow. This indicated that branching, *i.e.* reactions of PCL with the activated ester, did not take place or only in a very limited extent. It demonstrated that the NHS-activated ester survived the applied polymerization conditions. However, after a heating time of 12 hours the NHS group participated in the reaction. The blocked isocyanate groups were apparently more reactive than the NHS-ester. When the concentration of the blocked isocyanates group decreased during the

polymerization the NHS-ester became involved, resulting finally in a crosslinked polymer network. Since we were aiming for amorphous polyurethane networks, this crosslink reaction offered an important tool for preparing polyurethane networks.

Table 5.1 Molecular weights of PU-NHS, based on BLDI-NHS and PCL530, at various polycondesation temperatures and times

T(^{o}C)	Time(h)	M_n (Da)	M_w (Da)	PDI
130	4	4,850	7,650	1.57
	8	8,230	43,130	5.24
	12	Crosslinked		
135	4	6,370	14,780	2.32
	8	12,000	323,480	26.94
	12	Crosslinked		
140	4	6,920	7,340	1.49
	8	9,180	49,480	5.34
	12	Crosslinked		
145	4	8,780	21,070	2.40

The polymer obtained from the bulk polymerization of BLDI-NHS and PCL530 at 145 ^{o}C for 4 hours (with a M_n of 8,780 and a DPI 2.40) was chosen to be characterized in more detail and was used for further modifications. The structure was confirmed by ^{1}H-NMR spectroscopy, as depicted in Figure 5.1. It is important to stress that the signals of the NHS-group still were present (2.8 ppm; Chapter 2, Figure 2.6), confirming that the activated ester remained unaffected. Activated NHS ester groups can in principle react with all kind of nucleophiles, which would be useful to attach for instance the N-terminus of peptides or proteins, forming a stable covalent amide bond. As described in Chapter 2, in a model reaction the substitution of the NHS-group by particularly amines (hexyl amine) was successfully performed with the monomer.

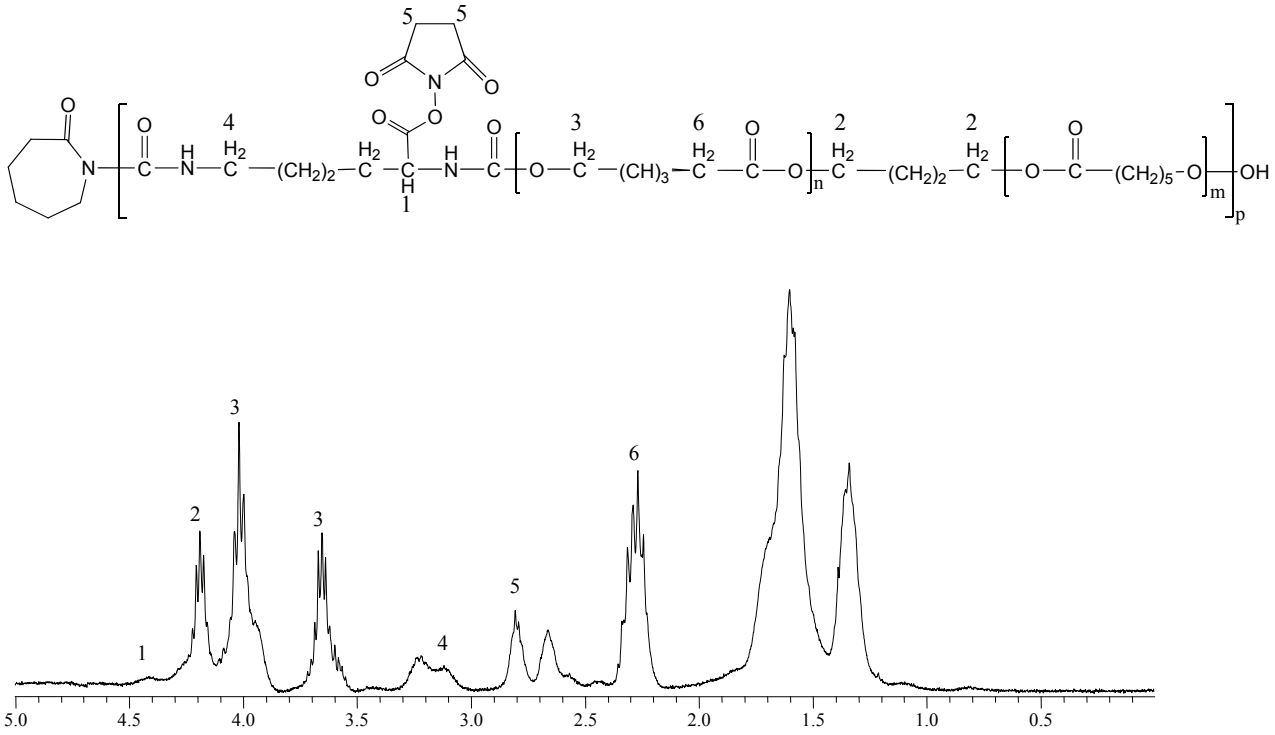


Figure 5.1 ^{1}H-NMR spectrum ($CDCl_3$) of the product obtained from the bulk polymerization of BLDI-NHS and PCL530 at 145 ^{o}C for 4 hours with a M_n of 8,780 Da and a DPI of 2.40.

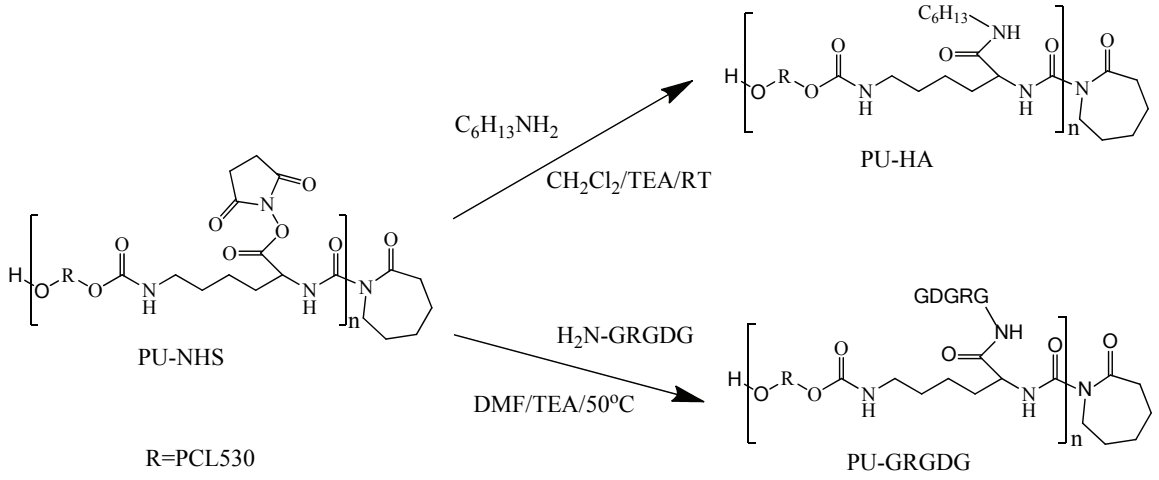
First the substitution of the NHS group of the pedant ester in the polymers by hexylamine, as a model compound, was studied to examine the possibility for introducing other functionalities. As our aim was to promote cell adhesion, the next step was to introduce a GRGDG peptide.

5.3.2. Modification of PU-NHS with hexyl amine and GRGDR

The polymer modification reaction is depicted in scheme 5.2. To prevent hydrolysis of NHS by traces of water anhydrous methylene chloride and DMF were used as solvent.[31] Hexylamine was indeed able to substitute the NHS group, thereby being coupled onto the polyurethane backbone, forming PU-HA, while a precipitate of NHS-amine salt was formed at same time. The reaction was performed at room temperature and monitored by

^{1}H-NMR via the disappearance of NHS signal and appearance of CH_3 of hexylamine signals at 2.8 and 0.8 ppm respectively (Figure 5.2). The reaction was complete after about 24 h. This indicates that the NHS groups of PU-NHS were able to be substituted by amino groups at room temperature, without affecting the ester bonds in the polymer backbone. This demonstrated a remarkable difference in sensitivity of the NHS-ester bond for amines and hydroxyl groups. While the hydroxyl groups of PCL hardly reacted with the NHS-ester at 145 ^oC, the amino group of hexyl amine reacted even at room temperature. These mild conditions were very important to prevent chain scission of the polyester backbone by amines.

Although the amino groups of GRGDG were less nucleophile that the primary amine of hexyl amine, we found that the substitution reaction proceeded as well. The reaction was performed at 50^oC in anhydrous DMF because of limited solvability of the peptide in DMF at room temperature. The GRGDG peptide substituted the NHS groups, while being coupled onto the polyurethane backbone forming PU-GRGDG and a precipitate of NHS-amine (TEA) salt at same time. The reaction was monitored by ^{1}H-NMR via the disappearance of NHS signal at 2.8 ppm.



Scheme 5.2 Synthesis of PU-HA and PU-GRGDG from PU-NHS

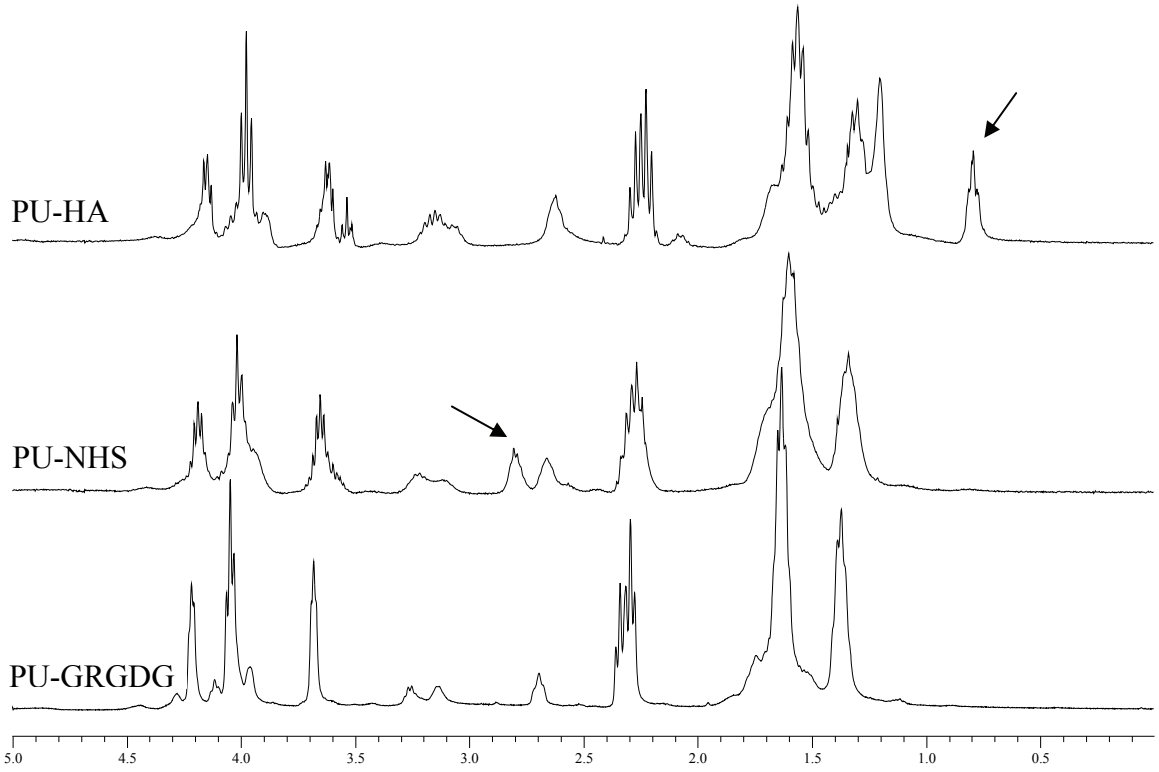
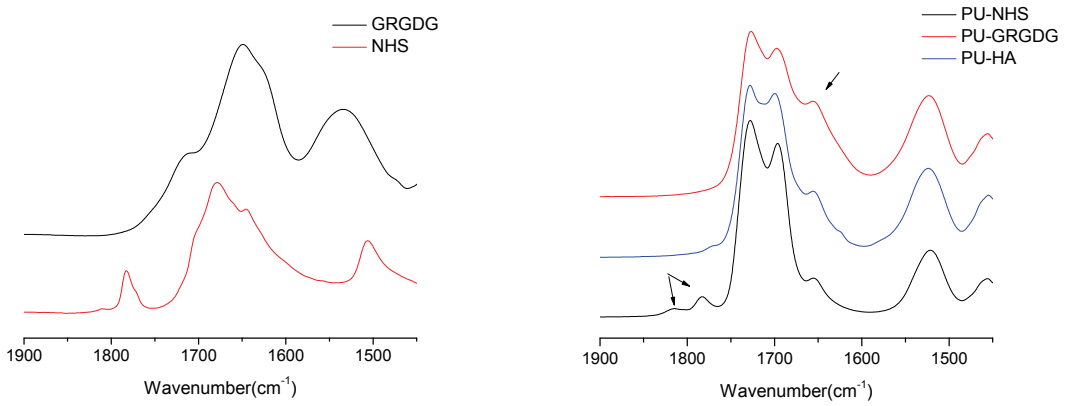


Figure 5.2 ^{1}H-NMR spectrum ($CDCl_3$) of PU-NHS, which was obtained from the bulk reaction at 145 ^{o}C for 4 hours from BLDI-NHS and PCl530 (M_n = 8,780 and DPI = 2.40) and the hexyl amine and GRGDG modified PUs.

The ^{1}H-NMR spectra indicated that a reaction with GRGDG took place, by the disappearance of the NHS moiety. However, the presence of the peptide was less well detectable with ^{1}H-NMR in $CDCl_3$. To gather more evidence, the modified polyurethanes were characterized by IR spectroscopy. Figure 5.3(a) shows the FTIR spectra of GRGDG and NHS in the region between 1900 and 1450 cm^{-1}. The spectra of PU-NHS, PU-GRGDG and PU-HA are depicted in Figure 5.3 (b). Strong absorbance bands were observed at 1715 cm^{-1} in the amide I (1630-1690 cm^{-1}) and amide II (1475-1585 cm^{-1}) regions of the GRGDG spectrum. In the NHS spectrum, the absorbance at 1783 cm^{-1} and 1815 cm^{-1} were assigned to carbonyl stretching of the N-hydroxysuccinimide (NHS) ring,[32] which were totally absent in PU-HA and PU-GRGDG spectra, as shown in Figure 5.3 (b). For all polymers, the bands at 1695 cm^{-1} and 1730 cm^{-1} were attributed to the urethane carbonyl and ester absorbance[23], respectively. The amide I bands partially

overlapped with the carbonyl stretching (1715 cm^{-1}) of polyurethanes. The important difference in these spectra is the increase of the intensity at approximately 1655 cm^{-1} for the PU-GRGDG sample. Since the spectrum of GRGDG showed strong bands in the same region, the increased intensity in the spectrum of modified polyurethane was attributed to the grafted peptide.[18] The differences between the spectra of PU-NHS, PU-HA and PU-GRGDG indicated that the NHS groups were successfully substituted by hexyl amine and GRGDG peptide, but further evidence was desired.



(a) (b)

Figure 5.3 ATR-FTIR spectra of (a):NHS and GRGDG; (b): PU-NHS, PU-HA and PU-GRGDG

5.3.2. Sakaguchi assay and Elemental analysis

To support the spectroscopic results, a chemical assay (Sakaguchi assay) and elemental analysis were performed. The Sakaguchi assay was used to verify the substitution reaction of the NHS group by the GRGDG peptide. In the Sakaguchi assay a solution will color orange on adding α-naphthol if arginine comprising peptides are present. Indeed, for the PU-GRGDG sample, an orange coloration (*i.e.* positive response) was observed, whereas the PU-NHS and PU-HA samples showed a negative response (Figure 5.4). Since arginine was detectable by the Sakaguchi reaction,[33] the results confirmed the coupling of peptide to the polyurethane.

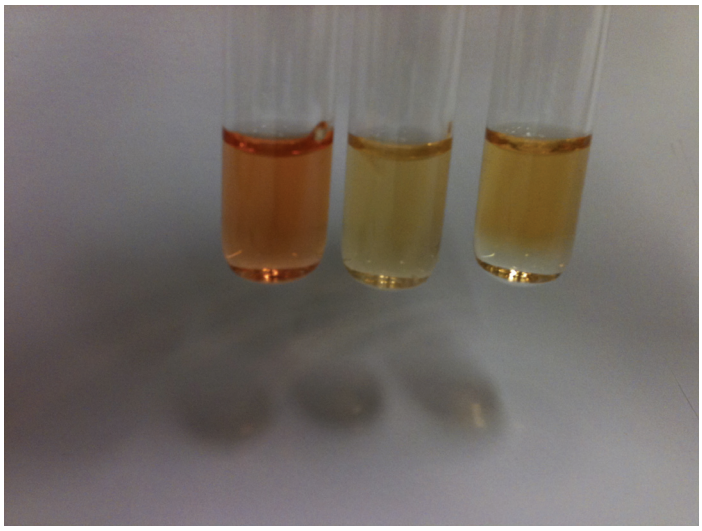


Figure 5.4 Results of Sakaguchi assay, from left: PU-GRGDG, PU-NHS, PU-HA

The success of the synthesis of the RGD-containing peptide grafted polyurethane was further investigated by elemental analysis. Table 5.2 summarizes the theoretical and experiment results of the nitrogen elemental analysis for polymers and GRGDG peptide.

Table 5.2 Elemental analysis: especially nitrogen content of PUs and GRGDG

PU	*N (wt%)*	
	Calculation value	*Experimental value*
PU-NHS	*5.28*	*5.45*
PU-HA	*5.35*	*5.45*
PU-GRGDG	*7.32*	*7.21*
GRGDG	*24.34*	*23.47*

When PU-NHS was modified by hexyl amine, there was only a slightly change of nitrogen content of the polymer, both in theoretical and experiment results. If every NHS group was substituted by GRGDG peptide, the nitrogen content should increase

substantially, from 5.28wt% to 7.32wt%, because the high nitrogen content of GRGDG peptides (24wt% N). The experimental PU-GRGDG sample contained 7.21% nitrogen which was very close to the theoretical value (7.32%). Based on the elemental analysis, it was calculated that modified polymer contained approximately 350mg of peptide per gram of PU-GRGDG. This quantitative analysis suggests that the substitution of the NHS groups on the polyurethane by the GRGDG peptide was nearly 100%.

5.3.3. Glass Transition temperature (T_g) of PU-NHS and modified PUs

Both the starting polymer and modified polymers are transparent sticky wax-like, slightly yellow transparent products. This matched with our targets, as we were aiming for low molecular, non-crystalline, potentially crosslinkable polymers. PU-GRGDG is a little stiffer than the others because of the high amount of hydrogen bonding moieties, introduced by the grafted peptide. The fully transparency indicated the absence of crystallization. Consequently, it was expected that our PUs would not have detectable melting temperatures. To verify this and to measure the glass transition temperatures (T_gs) of our PUs, DSC measurements were performed. The samples were heated from -60^{o}C to 90^{o}C at heating rate of 10^{o}C/min. In the DCS diagram, indeed, no sign of any crystallization peak was visible. Table 5.3 gives the glass transition temperatures (T_gs) of PU-NHS, PU-HA and PU-GRGDG.

Table 5.3 Glass transition temperature (T_g) of polyurethanes

PU	$T_g{}^oC$
PU-NHS	-30.2
PU-HA	-31.3
PUGRGDG	-22.9

All the samples showed the expected low T_g values because of the flexible long alkyl aliphatic polyester spacer segment (PCL530). All PUs had same soft segment, having the same chain mobility, which resulted in similar T_gs. It is worthy to note that the T_g of PU-

GRGDG was around 8°C higher than other samples. It can be attributed to the introdution of extra hydrogen bonding moieties by grafted peptide on the polyurethane.

5.3.4. Surface properties (XPS & water contact angle)

To determine contact angles and to analyze the materials by XPS the polymers were applied as coatings on glass slides. The polymers were dissolved in DMF (5% w/v), spin coated (2,000rpm) on glass substrates and subsequently dried under vacuum for 24h at 40°C.

The water contact angle measurement results are listed in Table 5.4. The glass slide and PU-OMe (sample as described in chapter 3) were chosen as the blank for comparison, since the NHS group of PU-NHS was suspected to be sensitive for hydrolysis in water.[31] The glass slide ($\alpha = 80^{o}$) and PU-OMe surface ($\alpha = 89^{o}$) appeared to be hydrophobic. In spite of the presence of the hydrophobic hexyl chain, the water contact angle of PU-HA was lower ($\alpha = 65^{o}$) than the blank samples. This can be attributed to the high content of amides, resulting in hydrogen bonding between polymer and water. Incorporation of the peptide onto the polymer backbone strongly decreased the contact angle to below 20° (Figure 5.5), and made the surface highly hydrophilic. This was obviously due to the high density of grafted water soluble GRGDG peptide on the surface.[25] This was further proved by the XPS measurement.

Table 5.4 Water Contact angles of various PUs

Sample	Water contact angle(°)
Glass slide	79.5 ± 3
PU-OMe	88.5 ± 3
PU-HA	64.6 ± 3
PU-GRGDG	17.7 ± 3

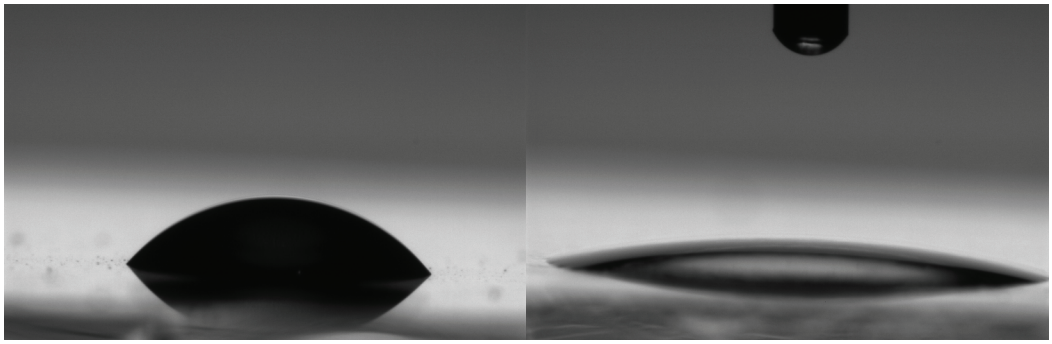


Figure 5.5 Pictures of a water droplet deposited on the surface of left: PU-HA; right: PU-GRGDG

The XPS analysis was used to determine the chemical composition of the surface layer, quantified by the N, C and O atomic percentages of PU-NHS and PU-GRGDG. In Figure 5.6 the XPS spectra of the PU-NHS and PU-GRGDG are shown. From these XPS spectra the elemental compositions of the surface layers were calculated. XPS measures about a 5-10 nm thick top layer of a surface. After modification of the polymer with the GRGDG peptide, the nitrogen content increased from 4.94 to 7.34wt%, which is quite similar as the bulk elemental analysis results, as shown in Figure 5.6 (a) and (c). It indicated that the density of GRGDG peptide on the surface was also around 350mg/g polymer, just the same as in the bulk. The C_{1s} peaks (Figure 5.6 (b) and (d)) were composed of the C-C component at 284.5eV due to aliphatic hydrocarbons, the C-N component at 285.64eV, the C-O component at 286.18eV, the O=C-N component at 286.68eV and O=C-O component at 288.6eV from urethane, amide and ester groups respectively.[34] Table 5.5 summarises the various chemical compositions and the atomic percentages for PU-NHS and PU-GRGDG. The peaks for carbon next to nitrogen in the form of C-N and O=C-N showed an increase from 12.73% and 3.21% to 14.52% and 5.72% respectively, after substitution of the NHS by the peptide.[35] This again strongly confirmed the successful attachment of nitrogen-rich peptides moieties to the polymer and it showed that the peptides were present on the surface in almost the same density as in the bulk of the polymer.

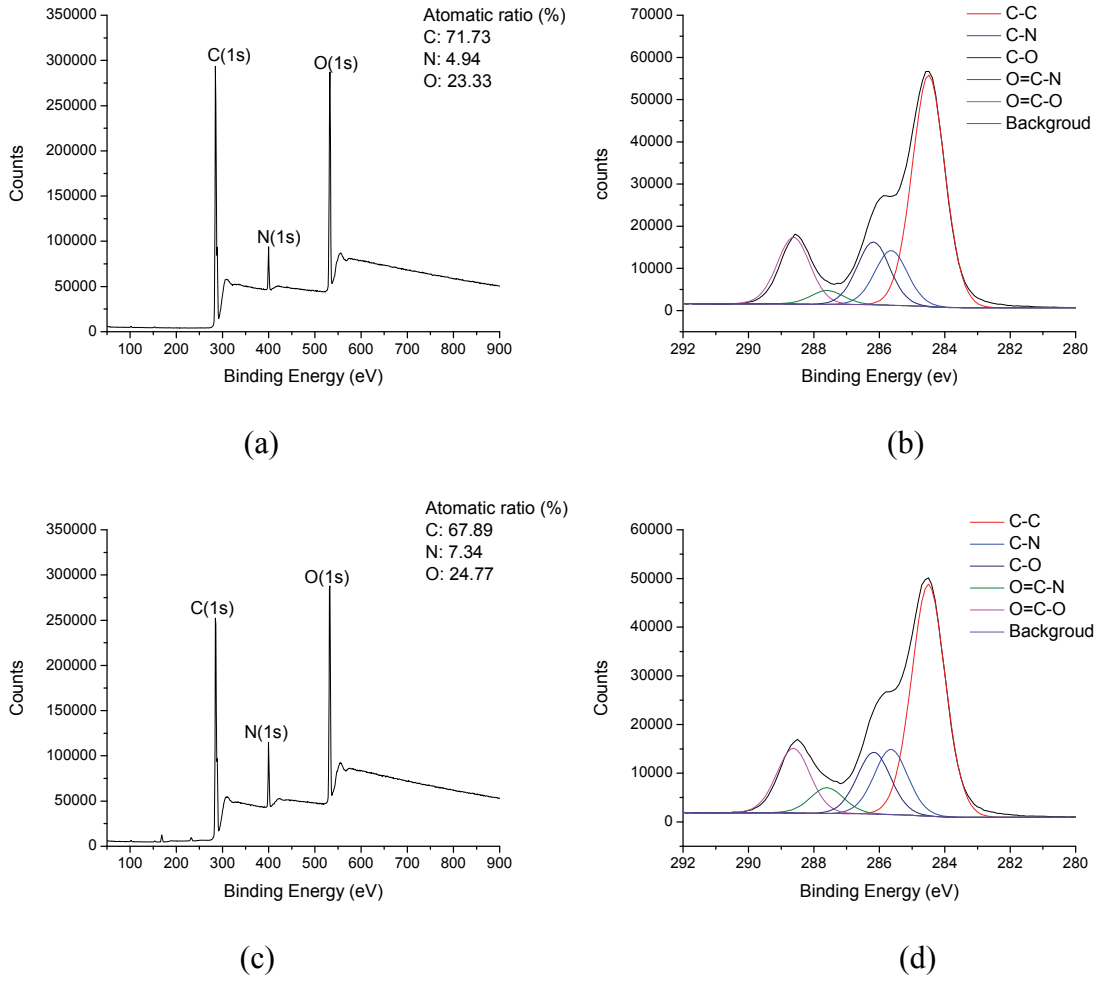


Figure 5.6 XPS spectra of (a) PU-NHS wide scan (b) PU-NHS C_{1s} core level region (c) PU-GRGDG wide scan (d) PU-GRGDG C_{1s} core level region

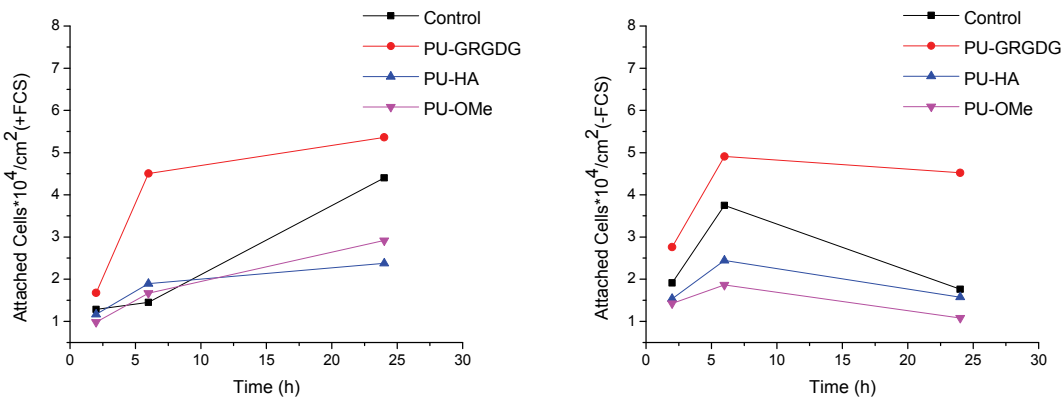
Table 5.5 Carbon 1s chemical state quantification by XPS of PU-NHS and PU-GRGDG (atomic percentages and the corresponding binding energies in eV)

Sample	C-C 284.5eV	C-N 285.64eV	C-O 286.18eV	O=C-N 286.68eV	O=C-O 288.6eV
PU-NHS	53.87%	12.73%	14.65%	3.21%	15.54%
PU-GRGDG	51.60%	14.52%	13.74%	5.72%	14.41%

The water contact angle data, supported by the XPS data, showed that the grafted peptides significantly increased the hydrophilicity of the coated surface, influencing strongly the surface properties of the polymers. This might improve the biocompatibility, which was studied by cell adhesion experiment.

5.3.5. Cell adhesion

Cell adhesion experiments on the polymer surface were an important test for evaluation of biocompatibility of polymers. Here we examined whether the peptides incorporated into polymer backbone could enhance Human dermal fibroblast cells (HDF) adhesion. PU-OMe was used as a blank, instead of PU-NHS, because of the water sensitivity of the latter. Figure 5.7 show the cell attachment profiles of the polyurethanes and, for control, the tissue culture polystyrene (TCPS) substrates in a serum (Figure 5.7 (a)) and a serum-free (Figure 5.7 (b)) medium.



(a) (b)

Figure 5.7 Human dermal fibroblast cell attachment profiles of TCPS, PU-GRGDG, PU-HA and PU-OMe: (a) EMEM containing 10% FCS; (b) EMEM without 10% FCS

After 2 hours of incubation, relatively small number of cells (about 10,000 cells/cm^2) were attached to all the substrates in the serum containing medium. The number of attached cells on PU-GRGDG was slightly higher (around 15,000 cells/cm^2) than on the

other polymers. Cell adhesion increased in a time-dependent fashion in the serum-supplemented media (Fig. 5.7(a)). After six hours, the adhesion on PU-GRGDG was a threefold (around 45,000 cells/cm^2) higher than on the other polymers (around 15,000cells/cm^2). After 24 hours, there were more cells attached on all substrates in the serum mediated growth experiments. Still the number of attached cells (around 55,000 cells/cm^2) was the highest on PU-GRGDG substrate. Adhesion of HDF onto PU-OMe and PU-HA was substantially lower (around 25,000 cells/cm^2). The adhesion properties of these two substrates did not differ significantly from each other. Apparently, GRGDG peptide modified polyurethane was more favorable to attached human cells, particularly in short times.[25,31] Other RGD comprising polymer systems that are well described in the literature, show similar performances.[31,32,34,35]

Without serum the cells survive for several hours until maximally several days, depending on the type of cells. We also performed cell adhesion experiments using a serum-free medium, since serum could influence the cell adhesion. Because serum contains fibronectin, which rapidly binds to almost any surface, rendering almost any surface adhesive to cells, but in a non-covalent fashion. Without serum, we could study the neat biomaterials properties much better, without the interference of other RGD-containing molecules (e.g. fibronectin). From Figure 5.7 (b) it can be seen that the number of all attached cells increased when the incubation time was raised from 2 hours to 6 hours. PU-GRGDG still attracted approximately two times more cells (around 50,000 cells/cm^2) than PU-HA and PU-OMe, and 1.5 times more than TCPS, after 6 hours incubation time. The adhesion had significantly decreased on all surfaces (in 0% FCS) except PU-GRGDG which still adhered around 46,000cells/cm^2 on the surface. This number is almost a fourfold of that of the other substrates. Obviously, a lot of cells died without serum after several hours, and, as a result, they were not able any more to attach to the surface of substrates. But, the adhesive peptides of PU-GRGDG maybe perform as the nutrition for cells, keeping them still live when attached on the polymer surface. It means GRGDG peptide modified polyurethane can attract not only more cells than unmodified polyurethanes in the serum-free circumstance, but keep them in addition longer alive.

The results suggested that the grafted GRGDG peptide of PU-GRGDG promoted Human dermal fibroblast cells (HDF) adhesion on the surface of polyurethane. It means that GRGDG peptides were assessable for bio-active interactions after coupling onto PU-NHS. Considering that NHS group can easily substituted by amino functional nucleophiles, PU-NHS can give numerous possibilities to synthesize a library of functional polyurethanes.

5.4. Conclusion

NHS-activated ester containing polyurethanes were successfully synthesized from NHS-functional caprolactam blocked lysine diisocyanate (BLDI-NHS) and PCL. During the polymerization the pendant NHS groups were not substituted by the hydroxyl groups of polycaprolactone (PCL), under well selected conditions, while they were easily substituted by amino functional compounds. Hexyl amines, as a model compound, as well as a bio-active amino-functional GRGDG peptide, were coupled onto the polymer backbone via the same route. ^{1}H-NMR, IR, XPS and elemental analysis showed almost 100% substitutions of the NHS-group by hexyl amine and the peptide, forming PU-HA and PU-GRGDG. XPS and elemental analysis of PU-HA- and PU-GRGDG proved that there was about 350mg peptide per gram polymer. According to XPS, the concentration of peptides on spin-coated surfaces was the same as in the bulk of the polymer. The low contact angle ($\alpha = 20^{o}$), due to the presence of GRGDG, was a further prove for the presence of the peptides on the surface. The GRGDG peptides promoted adhesion of HDF cells, even in the serum-free conditions. Since these polyurethanes could also be applied by a (spin) coating technique, they may also be used as functional coatings on the implantable devices to promote cell adhesion. Hence, PU-NHS can offer a platform to prepare a library of bio-functional polyurethanes.

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Chapter 6

Polyurethanes based on caprolactam blocked lysine diisocyanates decorated with biotin moieties

Abstract

The aim of this chapter was to explore the possibility for introducing a biotin moiety, with and without a long nontoxic spacer, onto our homemade blocked lysine diisocyanate and to synthesize corresponding polyurethanes. The presence of biotin on polymers would allow immobilization of various biomolecules, like peptides or protein, through biotin-avidin interactions. In a first step the pendant ester group of blocked lysine diisocyanate was converted into a primary amino or hydroxyl group. The pendant amino group was used to couple biotin and, as a result, biotinylated blocked lysine diisocyanate (BLDI-C_6-biotin) was obtained. The pendant hydroxyl group was nosylated by 4-nitrobenzenesulfonyl chloride, forming BLDI-C_6-ONs. This compound was used as an initiator for the ring opening polymerization of oxazolines. A primary amino group at the end of BLDI-C_6-PMOX was obtained by end-capping the living polymer with hexamethylene tetramine. Next, biotin was coupled onto the BLDI-PMOX, to form a BLDI-PMOX-biotin. The polymerization of the biotinylated blocked lysine diisocyantes with PCL530 yielded the corresponding polyurethanes.

6.1. Introduction

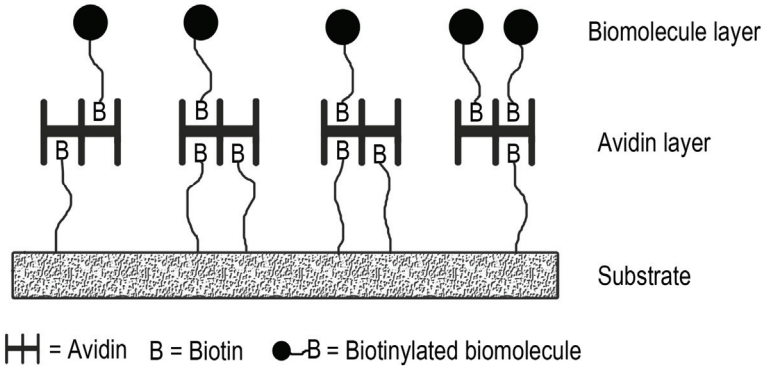
A number of polymers have proven their value as biomaterials because of the versatility in chemistry, which gives rise to materials with great diversity of physical and mechanical properties.[1] Depending on the application either long-lasting or temporarily properties are needed. Degradable polymers are of utmost interest for temporarily applications, such as in tissue engineering, as they are able to degrade and to resorb and excrete without surgical intervention. In this class of polymers, polyurethanes (PU) represent a major class of synthetic elastomers that have been evaluated for a variety of medical implants, particularly for long-term implants.[2,3] They have excellent mechanical properties and good biocompatibility. They are used in the fabrication of medical implants such as cardiac pace makers and vascular grafts. The main challenge is currently the inadequate interaction between these polymers and human cells, leading *in vivo* to foreign body reactions, such as inflammation, infections, aseptic loosening, local tissue waste, and implant encapsulation as well as thrombosis and embolization.[4,5] So far, modification of polymers with various biomolecules was chosen to solve this problem.

An attractive alternative to the biomolecule entrapment involves the covalent binding of the biomolecule to polymer films bearing adequate functional groups. Covalent bonds are mostly formed between side-chain-exposed functional groups or proteins with suitable modified supports. However, tissue engineering requires degradable polymers and the number of degradable polymers decorated with side groups are rare. Another handicap of the existing polymers is that often an irreversible binding is used to fix bio-active groups onto polymer backbones. Biochemical affinity (*i.e* avidin-biotin) reactions offer a gentle method for oriented immobilization of biomolecules, providing an important advantage over other immobilization techniques. Moreover, not only oriented and homogeneous attachment can be obtained but it is also possible to detach biomolecules and to make repeated use of the same surface.

The use of avidin-biotin technology in generating biocompatible surfaces has been reviewed in great detail.[6] The approach exploits one of the strongest non-covalent bonds ($K_d = 10^{15}$ M^{-1}) ever observed, preventing the use of harsh conditions during covalent binding biomaterials. An excellent overview of the binding couple avidin-biotin can be found in the book “Avidin-Biotin Chemistry: A Handbook”.[7] Avidin is a tetrameric

glycoprotein soluble in aqueous solutions and stable over wide pH and temperature ranges. It can bind up to four molecules of biotin. The bond formation is very rapid and unaffected by pH, temperature, organic solvents, enzymatic proteolysis, and other denaturing agents. Streptavidin is a closely related tetrameric protein, with similar affinity to biotin, but differing in other aspects, such as molecular weight, amino acid composition and so on. The properties of avidin and streptavidin have been improved using chemical and recombinant methods providing enhanced stability and/or controlled biotin binding. For example, NeutrAvidin displays highly specific binding to biotin, whereas NitrAvidin gives a pH-dependent biotin binding with strong interaction at acidic conditions (pH 4-5) and dissociation at a higher pH.

Biotin or vitamin H is a naturally occurring vitamin found in all living cells. Only the bicyclic ring is required to be intact for the interaction with avidin; the carboxyl group on the valeric acid side chain is not involved and can be modified to generate biotinylation reagents used for conjugation with proteins. Since biotin is a small molecule, its conjugation to macromolecules does not affect their conformation, size, or functionality. Biotinylation reagents can be classified depending on their reactivity toward diverse functional groups. The NHS ester of biotin is the most commonly used biotinylation reagent to target amine groups,[8] whereas biotin hydrazide can be used to target either carbohydrates or carboxyl groups.[9] A typical biotin/avidin/biotin multilayer is composed by biotin directly immobilized and avidin creating a secondary layer for binding biotinylated molecules (Scheme 6.1).



Scheme 6.1 Immobilization of biotinylated biomolecules to the substrate via avidin-biotin mediated systems.

This approach is generally preferred due to the higher organization obtained in comparison to that of the direct immobilization of avidin. For instance, the biotin layer promotes an ordered avidin over-structure with two biotin-binding sites facing the surface and the other two facing outward. A glycopolymer-derivatized surface over the biotin-streptavidin-biotin layer has been used for surface glycoengineering.[10]

Coupling of biotin groups to our polyurethanes would allow immobilizing of biomolecules, like peptides or protein, through biotin-avidin interactions. As described earlier (Chapter 2), the caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe) was successfully synthesized in high yields and the methylester could be hydrolyzed under well-defined conditions without affecting the blocked isocyanate group. The pendant acid groups was available to be converted into activated esters (chapter 2), which could offer the possibility to introduce biotin moieties. In this chapter, the synthesis of the biotinylated blocked lysine diisocyanate with and without space are reported. After that these functionalized blocked lysine diisocyanate were used to synthesize the corresponding functional polyurethanes.

6.2. Materials and Methods

6.2.1. Materials

CBC (carbonyl biscaprolactam) was obtained from DSM Innovation Center, ALLINCO®, (> 99 % pure according to HPLC) and used without purification.

L-Lysine monohydrochloride, thionylchloride, calciumchloride dihydrate, 1,6 hexanediol, hexamethylenediamine, 6-aminohexanol, 4-nitrobenzenesulfonyl chloride (NsCl), hexamethylenetetramine (HMTA), ninhydrin, 2-methyl-2-oxazoline (MOX) anhydrous magnesium sulfate, dicyclohexyl-carbodiimide (DCC), N-hydroxysuccinimide (NHS), biotin, chloroform-d ($CDCl_3$-D, 99.8 atom % D) and dimethyl sulfoxide-d_6 (DMSO-d_6, 99.5 atom % D) were purchased from Sigma-Aldrich and used as obtained.

Ethyl acetate, potassium hydroxide (KOH), sodium hydroxide (NaOH), anhydrous acetonitrile and anhydrous *N,N*-dimethylformamide (DMF), were purchased from Acros Organics and used as obtained.

Methanol, acetone, chloroform, triethylamine (TEA), n-hexane, hydrochloric acid fuming 37%, sulfuric acid, tetrahydrofuran (THF), dichloromethane (DCM), diethyl ether, were purchased from Lab-Scan Analytical Science and used as obtained.

Caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe), the corresponding acid (BLDI-OH) and NHS modified acid **(**BLDI-NHS) were synthesized according to the method as described previously (chapter 2).

6.2.2. Analysis

Column chromatography was performed on silica gel (Silica-P flash silica gel from Silicycle, size 40-63 μm). TLC was performed on silica gel 60/Kieselguhr F254. Components were visualized by UV and staining with a solution of a mixture of $KMnO_4$ (5g) and K_2CO_3 (20 g) in H_2O (500 mL). The presence of primary amines in NH_2-containing compounds were detected by using ninhydrin.

^{1}H-NMR was recorded on a Varian AMX400 using $CDCl_3$ or DMSO-d_6 as solvent. Chemical shift values are reported in ppm with the solvent resonance as the internal standard ($CHCl_3$: δ 7.26 for ^{1}H; d_6-DMSO: δ 3.3 and 2.5 for ^{1}H). Data are reported as follows: chemical shifts, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet).

Mass spectra were recorded on a LTQ Orbitrap XL (ESI+).

Size exclusion chromatography (SEC) measurements were performed in dimethylformamide with 0.01M LiBr on a Viscotek GPC max equipped with model 302 TDA detectors, using 2 columns (Pl-gel 5μ 30 cm mixed-C from Polymer Laboratories). The data analysis was done using conventional calibration with polystyrene standards accompanied by in-house software.

ATR-FTIR spectra were recorded on a Broker IFS88 spectrometer equipped with a Golden Gate (Graseby specac) single reflection ATR accessory. Spectra resolution was 4 cm^{-1} and 32 scans were taken per spectrum.

The water contact angle measurements were carried out at room temperature by using a custom built microscope-goniometer system. A 1.00 μL drop of the deionized water was placed on a prepared polymer-coated glass slide using a Hamilton micro-syringe and the contact angle was measured immediately. The measurements involve fitting of a drop picture with home-built software. Three measurements were carried out in different regions on the surface.

6.2.3. Synthesis of BLDI-NH-C6-biotin

BLDI-NH-C_6-NH_2 (2). BLDI-NHS (1) (5g, 9.6mmol, from chapter 2) was dissolved in 100 methylene chloride in a glass flask of 250 mL in a nitrogen atmosphere. A 10 times molar excess of hexamethylene diamine (11.2g, 96mmol) was added. The reaction mixture was stirred at room temperature overnight. After removal of precipitated NHS salt by filtration, the crude product was washed with 500mL 5% $NaHCO_3$ aqueous solution and subsequently with 300mL brine (saturated NaCl aqueous solution). The resultant solution was dried over anhydrous $MgSO_4$. After filtering off $MgSO_4$ the solvent was evaporated in a rotavapor and further died at 45^oC for 48h in the vacuum oven.

^{1}H-NMR ($CDCl_3$): δ= 9.52 (1H, d, CHN<u>H</u>), 9.22 (1H, t, CH_2N<u>H</u>), 6.45 (1H, t, N<u>H</u>C_6H_{12}), 4.21 (1H, q, C<u>H</u>), 3.95 (4H, m, caprolactam ring NC<u>H_2</u>), 3.24 (4H, m, NHC<u>H_2</u>), 2.68 (4H, m, caprolactam ring C<u>H_2</u>CO), 1.95-1.25 (28H, m, caprolactam ring 6C<u>H_2</u> + alkyl chain 8C<u>H_2</u>).

BLDI-NH-C_6-biotin (3). N-hydroxysuccimide (1.32g, 11.3mmol) and dicyclohexylcarbodiimide (2.35g, 11.4mmol), were added to biotin (2.35g, 9.6mmol) dissolved in anhydrous 80 mL dimethylformammide (DMF) in a glass flask under nitrogen atmosphere. The reaction mixture was stirred for 24h at room temperature.[72] After dicyclohexyl urea was filtered off, BLDI-C_6-NH_2 (2) in DMF (5.15g, 9.5mmol in 20mL DMF) and triethylamine were added. After 4h, the NHS salt was filtered off. The solution was concentrated by rotary evaporator and precipitated in diethyl ether. After flash chromatography with chloroform and methanol with ratio of 4:1, the elute solvent was evaporated and the solid was dried at 50^oC for 2days in the vacuum oven, giving 3g (60%) biotinylated blocked lysine diisocyanate BLDI-NH-C_6-biotin.

^{1}H-NMR (DMSO-d_6): δ = 9.46 (1H, d, OCCHN<u>H</u>CO), 9.10 (1H, t, CH_2N<u>H</u>CO), 8.06 (1H, t, CH_2N<u>H</u>COCH), 7.74 (1H, t, CH_2N<u>H</u>COCH$_2$), 6.40 (1H, d, biotin ring: CON<u>H</u>CHCH), 6.34 (1H, d, biotin ring: CON<u>H</u>CHCH$_2$), 4.30 (1H, t, biotin ring: NHC<u>H</u>CH$_2$), 4.28 (1H, q, OCC<u>H</u>NHCO), 4.12 (1H, q, biotin ring: CONHC<u>H</u>CH), 3.86 (4H, m, NC<u>H</u>$_2$), 3.12-2.95 (7H, m, 3NHC<u>H</u>$_2$ + SC<u>H</u>), 2.78 and 2.56 (2H, m, biotin ring SC<u>H</u>$_2$), 2.65 (4H, m, C<u>H</u>$_2$CON), 2.18 and 2.02 (2H, NHCOC<u>H</u>$_2$), 1.87-1.21 (32H, m, caprolactam ring 6C<u>H</u>$_2$ + alkyl chain 10C<u>H</u>$_2$).

ESI-MS m/z: $(M+1)^+$ 751.06 $(M+Na)^+$ 773.04

6.2.4. Synthesis of Nosylate initiator (imide linkage)

BLDI-NH-C_6-OH (4). BLDI-NHS (1) (5g, 9.6mmol) was dissolved in 50 mL methylene chloride. An excess of 6-aminohexanol (2.3g, 19.6mmol) was added. The reaction mixture was stirred at room temperature overnight. After removal of precipitated NHS salt by filtration, the solution was washed with 300mL 5% $NaHCO_3$ aqueous solution and with 300mL brine (saturated NaCl aqueous solution). The resultant solution was dried over anhydrous $MgSO_4$. After removal of $MgSO_4$ by filtration and solvent by evaporation, the crude product was collected give around 4.5g (90%) BLDI-NH-C_6-OH.

^{1}H-NMR ($CDCl_3$): δ= 9.50 (1H, d, CHN<u>H</u>), 9.19 (1H, t, CH_2N<u>H</u>), 6.50 (1H, t, N<u>H</u>C_6H_{12}OH), 4.18 (1H, q, C<u>H</u>), 3.88 (4H, m, caprolactam ring NC<u>H</u>$_2$), 3.52 (2H, m,

C$\underline{H_2}$OH), 3.18 (4H, m, NHC$\underline{H_2}$), 2.64 (4H, t, caprolactam ring C$\underline{H_2}$CO), 1.95-1.22 (26H, m, caprolactam ring 6C$\underline{H_2}$ + alkyl chain 7C$\underline{H_2}$).

BLDI-NH-C_6-ONs (5). BLDI-NH-C_6-OH (4) (5g, 9.5mmol) was dissolved in 40mL of chloroform. 4-nitrobenzenesulfonyl chloride (NsCl) (2.53 g, 11.6mmol) was added drop wise to a solution of BLDI-NH-C_6-OH at 0 ^oC, followed by drop wise addition of TEA to the mixture solution. Reaction was carried out at 0 ^oC for 2 hours and another 4 hours at room temperature under nitrogen atmosphere. The obtained product was washed with 200mL 5% citric acid, 200mL 5% $NaHCO_3$ aqueous solution and with 300mL brine. The resultant solution was dried over anhydrous $MgSO_4$. After removal of $MgSO_4$ by filtration and solvent by evaporation, the crude product was collected. After flash chromatography with chloroform, the pure product was collected by evaporating the solvent by using the rotavapor to give around 4g (60%) BLDI-NH-C_6-ONs.
^{1}H-NMR ($CDCl_3$): δ= 9.51 (1H, d, CHN$\underline{H}$), 9.22 (1H, t, $CH_2N\underline{H}$), 8.39 (2H, d, benzyl ring C$\underline{H}$CNO_2), 8.19 (2H, d, benzyl ring C$\underline{H}$CSO_3), 6.25 (1H, t, N$\underline{H}C_6H_{12}O$), 4.20 (1H, q, C$\underline{H}$), 4.12 (2H, m, C$\underline{H_2}$O), 3.94 (4H, m, caprolactam ring NC$\underline{H_2}$), 3.24 (4H, m, NHC$\underline{H_2}$), 2.68 (4H, t, caprolactam ring C$\underline{H_2}$CO), 1.95-1.25 (26H, m, caprolactam ring 6C$\underline{H_2}$ + alkyl chain 7C$\underline{H_2}$).
ESI-MS m/z: $(M+1)^+$ 709.32, $(M+Na)^+$ 731.30.

6.2.5. Synthesis of poly(2-methyl-2-oxazolines) (BLDI-NH-PMOX-ONs) (6) and kinetic study

2-methyl-2-oxazoline (MOX) (3.7 mL, 43mmol) was mixed with 37 mL anhydrous acetonitrile in a nitrogen atmosphere and cooled to 0^oC. The initiator BLDI-NH-C_6-ONs (5) (3.04 g, 4.3mmol, amount calculated according to $[M_o]/[I]$ =10) was added to this mixture and left at 0 ^oC for 1 hour. The solution was then quickly heated to reflux and stirred for several hours till all MOX was consumed. The living polymer was collected after precipitating in diethyl ether and, after isolation by filtration, dried in vacuum oven at 40 ^oC for 48 hour. During the polymerization, samples were taken, once per hour to do the kinetic study, which were followed by ^{1}H-NMR measurement.

^{1}H-NMR ($CDCl_3$): δ= 9.52 (1H, d, CHN<u>H</u>), 9.24 (1H, t, CH_2N<u>H</u>), 8.24 (2H, d, aromatic ring C<u>H</u>CNO_2), 8.01(2H, d, aromatic ring C<u>H</u>CSO_3), 6.37 (1H, broad, N<u>H</u>$C_6H_{12}O$), 4.36 (1H, d, ring NC<u>H</u>CH_3), 4.24 (1H, q, C<u>H</u>), 3.95 (4H, m, caprolactam ring NC<u>H</u>$_2$), 3.72-3.28 (repeating unit of PMOX NC<u>H</u>$_2$-C<u>H</u>$_2$N), 3.24 (4H, m, NHC<u>H</u>$_2$), 2.68 (4H, t, caprolactam ring C<u>H</u>$_2$CO), 2.29-2.00 (repeat unit of PMOX CH_3), 1.95-1.25 (26H, m, caprolactam ring 6C<u>H</u>$_2$ + alkyl chain 7C<u>H</u>$_2$).

6.2.6. Synthesis BLDI-NH-PMOX-biotin

BLDI-NH-PMOX-NH_2. (7) The living polymer from 6.2.5 (3.6g, 2.4mmol) was terminated before precipitation by adding hexamethylenetetramine (HMTA, 0.84g, 6mmol) dissolved in 10mL chloroform. After 1 hour stirring at room temperature the solvent was evaporated. The residue was dissolved in 10 mL of toluene, 15mL methanol and 1.9g of concentrated sulfuric acid and solution was refluxed for 4 hours. The mixture was neutralized by adding a 50wt% NaOH/water solution, the salts were filtered off and the filtrate was stripped to dryness on a rotary evaporator. The residue was dissolved in chloroform and precipitated in diethyl ether. The white powder was further dried by vacuum oven at 40^{o}C for 48hour leaving around 2.2g (60%) of BLDI-NH-PMOX-NH_2.
^{1}H-NMR ($CDCl_3$): δ= 9.52 (1H, d, CHN<u>H</u>), 9.24 (1H, t, CH_2N<u>H</u>), 6.37 (1H, broad, N<u>H</u>$C_6H_{12}O$), 4.24 (1H, q, C<u>H</u>), 3.95 (4H, m, caprolactam ring NC<u>H</u>$_2$), 3.72-3.28 (repeating unit of PMOX NC<u>H</u>$_2$-C<u>H</u>$_2$N), 3.24 (4H, m, NHC<u>H</u>$_2$), 2.68 (4H, t, caprolactam ring C<u>H</u>$_2$CO), 2.29-2.00 (repeat unit of PMOX CH_3), 1.95-1.25 (26H, m, caprolactam ring 6C<u>H</u>$_2$ + alkyl chain 7C<u>H</u>$_2$).

BLDI-NH-PMOX-biotin. (8) N-hydroxysuccimide (NHS, 0.16g, 1.4mmol) and dicyclohexylcarbodiimide (DCC, 0.31g, 1.5mmol), were added to biotin that was dissolved in anhydrous dimethylformamide (0.32g, 1.3mmol in 20mL DMF) under N_2. The reaction mixture was stirred for 24h at room temperature.[72] The precipitated dicyclohexyl urea was filtered off. Subsequently, BLDI-NH-PMOX-NH_2 (7) in DMF (2.02g, 1.3mmol in 10mL DMF) and triethylamine (0.3g, 3.0mmol) were added. After

12h stirring at room temperature the solvent was evaporated. The residue was dissolved in 10mL chloroform, the salts were filtered off and the filtrate was precipitated in diethyl ether. The product was isolated by filtration and further dried in vacuum oven 40^{o}C for 48hour leaving around 1.5g (75%) of BLDI-NH-PMOX-biotin.

^{1}H-NMR (DMSO-d_6): δ= 9.52 (1H, d, CHN<u>H</u>), 9.24 (1H, t, CH_2N<u>H</u>), 6.40 (1H, d, biotin ring: CON<u>H</u>CHCH), 6.34 (1H, d, biotin ring: CON<u>H</u>CHCH_2), 4.21 (1H, t, biotin ring: NHC<u>H</u>CH_2), 4.06 (1H, q, OCC<u>H</u>NHCO), 3.96 (1H, q, biotin ring: CONHC<u>H</u>CH), 3.80 (4H, m, caprolactam ring NC<u>H</u>$_2$), 3.45-3.19 (repeating unit of PMOX NC<u>H</u>$_2$-C<u>H</u>$_2$N), 3.03 (4H, m, NHC<u>H</u>$_2$), 2.69-2.56 (7H, m, 3NHC<u>H</u>$_2$ + SC<u>H</u>), 2.71 and 2.51 (2H, m, biotin ring SC<u>H</u>$_2$), 2.45 (4H, m, caprolactam ring C<u>H</u>$_2$CO), 2.0-1.84 (repeat unit of PMOX C<u>H</u>$_3$), 1.64-1.16 (32H, m, caprolactam ring 6C<u>H</u>$_2$ + alkyl chain 10C<u>H</u>$_2$).

6.2.7. Synthesis BLDI-O-PMOX-biotin

BLDI-O-C_6-OH (10). BLDI-OH (9) (5g, 11.8mmol, from chapter 2) was dissolved in 50mL chloroform, whilst being cooled at 0^{o}C by using an ice bath. Thionyl chloride (1mL, 13.7mmol) was added drop wise whilst maintaining the temperature below 20^{o}C. A ten-fold excess of 1, 6-hexanediol (14g, 118mmol) was added. The reaction mixture was stirred at room temperature for another 48 hours. The solution was washed with 300mL 5% $NaHCO_3$ aqueous solution and with 300mL brine (saturated NaCl aqueous solution). The resultant solution was dried over anhydrous $MgSO_4$. After removal of $MgSO_4$ by filtration and solvent by evaporation, the crude product was collected. Then after flash chromatography with chloroform and methanol with ratio of 60:1, the pure product was collected to give around 6g (65%) BLDI-O-C_6-OH.

^{1}H-NMR ($CDCl_3$): δ= 9.66 (1H, d, CHN<u>H</u>), 9.21 (1H, t, CH_2N<u>H</u>), 4.40 (1H, q, C<u>H</u>), 4.09 (2H, m, COOC<u>H</u>$_2$) 3.92 (4H, m, caprolactam ring NC<u>H</u>$_2$), 3.48 (2H, m,C<u>H</u>$_2$OH), 3.22 (2H, m, NHC<u>H</u>$_2$), 2.68 (4H, t, caprolactam ring C<u>H</u>$_2$CO), 1.94-1.22 (26H, m, caprolactam ring 6C<u>H</u>$_2$ + alkyl chain 7C<u>H</u>$_2$).

BLDI-O-C_6-ONs (11). BLDI-O-C_6-OH (10) (5g, 9.5mmol) was dissolved in 40mL of chloroform. 4-nitrobenzenesulfonyl chloride (NsCl) (2.53 g, 11.6mmol) was added drop

wise to solution of BLDI-O-C_6-OH at 0 ^oC followed by addition of TEA drop wise to the mixture. Reaction was carried out at 0 ^oC for 2 hours and another 4 hours at room temperature. The obtained product was washed with 200mL 5% citric acid, 200mL 5% $NaHCO_3$ aqueous solution and with 300mL brine. The resultant solution was dried over anhydrous $MgSO_4$. After removal of $MgSO_4$ by filtration and solvent by evaporation, the crude product was collected. Then after flash chromatography with chloroform, the pure product was collected to give around 4g (60%) BLDI-O-C_6-ONs.

^{1}H-NMR ($CDCl_3$): δ= 9.69 (1H, d, CHN<u>H</u>), 9.25 (1H, t, CH_2N<u>H</u>), 8.39 (2H, d, aromatic ring C<u>H</u>CNO_2), 8.12 (2H, d, aromatic ring C<u>H</u>CSO_3), 4.38 (1H, q, C<u>H</u>), 4.15 (2H, m, C<u>H_2</u>O), 4.09 (2H, m, COOC<u>H_2</u>), 3.94 (4H, m, caprolactam ring NC<u>H_2</u>), 3.24 (2H, m, NHC<u>H_2</u>), 2.68 (4H, t, caprolactam ring C<u>H_2</u>CO), 1.95-1.25 (26H, m, caprolactam ring 6C<u>H_2</u> + alkyl chain 7C<u>H_2</u>).

ESI-MS m/z: $(M+1)^+$ 724.29, $(M+Na)^+$ 747.15.

BLDI-O-PMOX-ONs (12) and kinetic study. 2-methyl-2-oxazoline (MOX) (3.7 mL, 43mmol) was mixed with 37 mL anhydrous acetonitrile in a nitrogen atmosphere and cooled to 0^oC. The initiator BLDI-O-C_6-ONs (11) (3.04 g, 4.3mmol, amount calculated according to $[M_0]/[I] = 10$) was added to this mixture and the latter was left at 0 ^oC for 1 hour. The solution was then quickly heated to reflux and stirred for several hours till all the MOX was consumed. The living polymer was collected after precipitating in diethyl ether and after isolation by filtration dried in vacuum oven at 40 ^oC for 48 hour. During the polymerization, samples were taken after each hour, to do the kinetic study. The molecular weights were based on ^{1}H-NMR measurement.

^{1}H-NMR ($CDCl_3$): δ= 9.52 (1H, d, CHN<u>H</u>), 9.24 (1H, t, CH_2N<u>H</u>), 8.24 (2H, d, aromatic ring C<u>H</u>CNO_2), 8.01(2H, d, aromatic ring C<u>H</u>CSO_3), 4.36 (1H, d, ring N^+C<u>H</u>CH_3), 4.24 (1H, q, C<u>H</u>), 4.09 (2H, m, COOC<u>H_2</u>), 3.95 (4H, m, caprolactam ring NC<u>H_2</u>), 3.72-3.28 (repeating unit of PMOX NC<u>H_2</u>-C<u>H_2</u>N), 3.24 (2H, m, NHC<u>H_2</u>), 2.68 (4H, t, caprolactam ring C<u>H_2</u>CO), 2.29-2.00 (repeat unit of PMOX C<u>H_3</u>), 1.95-1.25 (26H, m, caprolactam ring 6C<u>H_2</u> + alkyl chain 7C<u>H_2</u>).

BLDI-O-PMOX-NH_2 (13). The living polymer (3.6g, 2.4mmol) was terminated by adding a hexamethylenetetramine (HMTA, 0.84g, 6mmol) solution (in 10mL chloroform). After 1 hour stirring at room temperature in nitrogen the solvent was evaporated. The residue was dissolved in 10 mL of toluene, 15mL methanol and 1.9g of concentrated sulfuric acid and the solution was reflux for 4 hours. The mixture was neutralized by adding 50wt% NaOH in water solution, the salts were filtered off and the filtrate was stripped to dryness on a rotary evaporator. The residue was dissolved in chloroform and precipitated in diethyl ether. The white powder was further dried by vacuum oven at 40^{o}C for 48hour leaving 2.2g (60%) of BLDI-O-PMOX-NH_2.

^{1}H-NMR ($CDCl_3$): δ= 9.52 (1H, d, CHNH), 9.24 (1H, t, CH_2NH), 4.24 (1H, q, CH), 4.09 (2H, m, COOCH_2), 3.95 (4H, m, caprolactam ring NCH_2), 3.72-3.28 (repeating unit of PMOX NCH_2-CH_2N), 3.24 (4H, m, NHCH_2), 2.68 (2H, t, caprolactam ring CH_2CO), 2.29-2.00 (repeat unit of PMOX CH_3), 1.95-1.25 (26H, m, caprolactam ring 6CH_2 + alkyl chain 7CH_2).

.

BLDI-O-PMOX-biotin(14). N-hydroxysuccimide (NHS, 0.16g, 1.4mmol) and dicyclohexylcarbodiimide (DCC, 0.31g, 1.5mmol), were added to biotin dissolved in anhydrous dimethylformamide (0.32g, 1.3mmol in 20mL DMF). The reaction was stirred for 24h at room temperature under nitrogen. Dicyclohexyl urea was filtered off, and BLDI-O-PMOX-NH_2 (13) in DMF (2.02g, 1.3mmol in 10mL DMF) and triethylamine were added. After 12h stirring at room temperature the solvent was evaporated. The residue was dissolved in 10mL chloroform, the salts were filtered off and the filtrate was precipitated in diethyl ether. After isolation by filtration the product was further dried in vacuum oven 40^{o}C for 48hour leaving 1.5g (75%) of BLDI-O-PMOX-biotin.

^{1}H-NMR (DMSO-d_6): δ= 9.47 (1H, d, CHNH), 9.07 (1H, t, CH_2NH), 6.34 (1H, d, biotin ring: CONHCHCH), 6.27 (1H, d, biotin ring: CONHCHCH_2), 4.20 (2H, m, COOCH_2), 4.07 (1H, t, biotin ring: NHCHCH_2), 3.96 (1H, m, OCCHNHCO), 3.96 (1H, q, biotin ring: CONHCHCH), 3.80 (4H, m, caprolactam ring NCH_2), 3.45-3.19 (repeating unit of PMOX NCH_2-CH_2N), 3.03 (4H, m, NHCH_2), 2.69-2.56 (7H, m, 3NHCH_2 + SCH), 2.71 and 2.51 (2H, m, biotin ring SCH_2), 2.45 (4H, m, caprolactam ring CH_2CO), 2.0-1.84

(repeat unit of PMOX C<u>H</u>$_3$), 1.64-1.12 (32H, m, caprolactam ring 6C<u>H</u>$_2$ + alkyl chain 10C<u>H</u>$_2$).

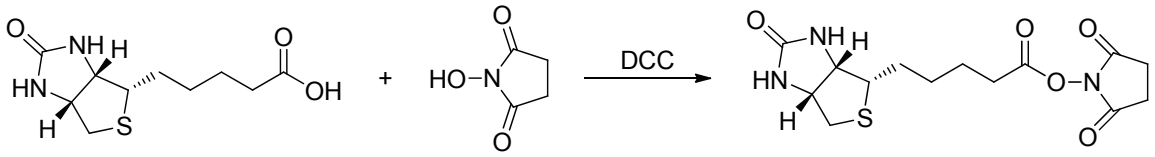
6.2.8. Synthesis of biotinylated polyurethanes

A 10 mL glass flask was fitted with a nitrogen inlet and an outlet to a vacuum pump. After the flask was three times evacuated and refilled with nitrogen, to remove all oxygen, dry biotinylated monomers BLDI-NH-C_6-biotin (3) or BLDI-NH-PMOX-biotin (8) or BLDI-O-PMOX-biotin (14) were added in an roughly equal molar with respect to PCL530, respectively. The mixture was heated up to 160 ^oC while stirring under vacuum for 24hours.

6.3. Results and discussion

6.3.1. Synthesis of BLDI-NH-C_6-biotin

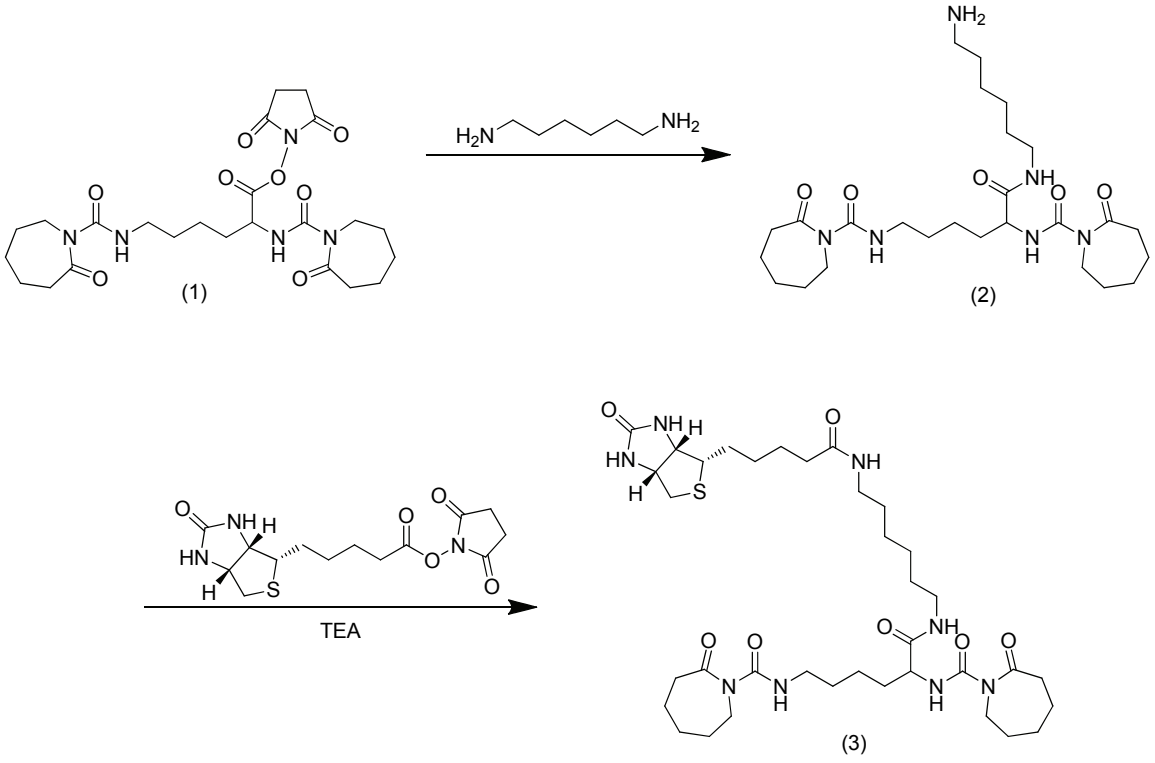
There are various options to incorporate biotin in polyurethanes. In one option the carboxylic acid group of biotin was converted with N-hydroxysuccimimide (NHS) into the corresponding reactive NHS-ester, according to the method described in the literature (Scheme 6.2).[11] The activated ester can easily react with primary amino groups.



Scheme 6.2 Synthesis of biotin-NHS[72]

The synthesis of BLDI-NHS has been reported in detail in chapter 2. It was shown that a model compound, hexylamine, was able to substitute the NHS group, thereby being coupled onto the ester group of blocked lysine diisocyanate, forming BLDI-HA. A precipitate of NHS-amine salt was formed at same time.

In order to prepare an amino functional BLDI (BLDI-NH-C_6-NH_2 (2)) a large excess of hexamethylene diamine was used (ten folds to blocked diisocyanate) to reduce the possibility that both amino groups would react with BLDI-NHS. The reaction was performed at room temperature and the disappearance of NHS signal and appearance of amide signals were monitored by ^{1}H-NMR at 2.82 and 6.45 ppm, respectively. The reaction was complete after about 12 h. After removing the excess of hexamethylene diamine and by subsequently adding biotin-NHS, biotin was coupled onto BLDI-NH-C_6-NH_2. After stirring for 12 hours at room temperature, the crude product was isolated by precipitating in diethyl ether and further purified using column chromatography. Scheme 6.3 shows the structure and the synthetic route to prepare the biotinylated blocked lysine diisocyanate (BLDI-NH-C_6-biotin).



Scheme 6.3 Synthesis of BLDI-NH-C_6-NH_2 and BLDI-NH-C_6-biotin

The reaction was monitored by ^{1}H-NMR and in figure 6.1 the ^{1}H-NMR spectrum of BLDI-NH-C_6-biotin is shown. The peaks at δ 6.40 and 6.34 ppm are belonging to the NH

from biotin ring which is very characteristic in ^{1}H-NMR spectrum of biotin.[12] All other signals in the spectrum were in agreement with the expected products. ESI-MS results supported that the desired product was obtained.

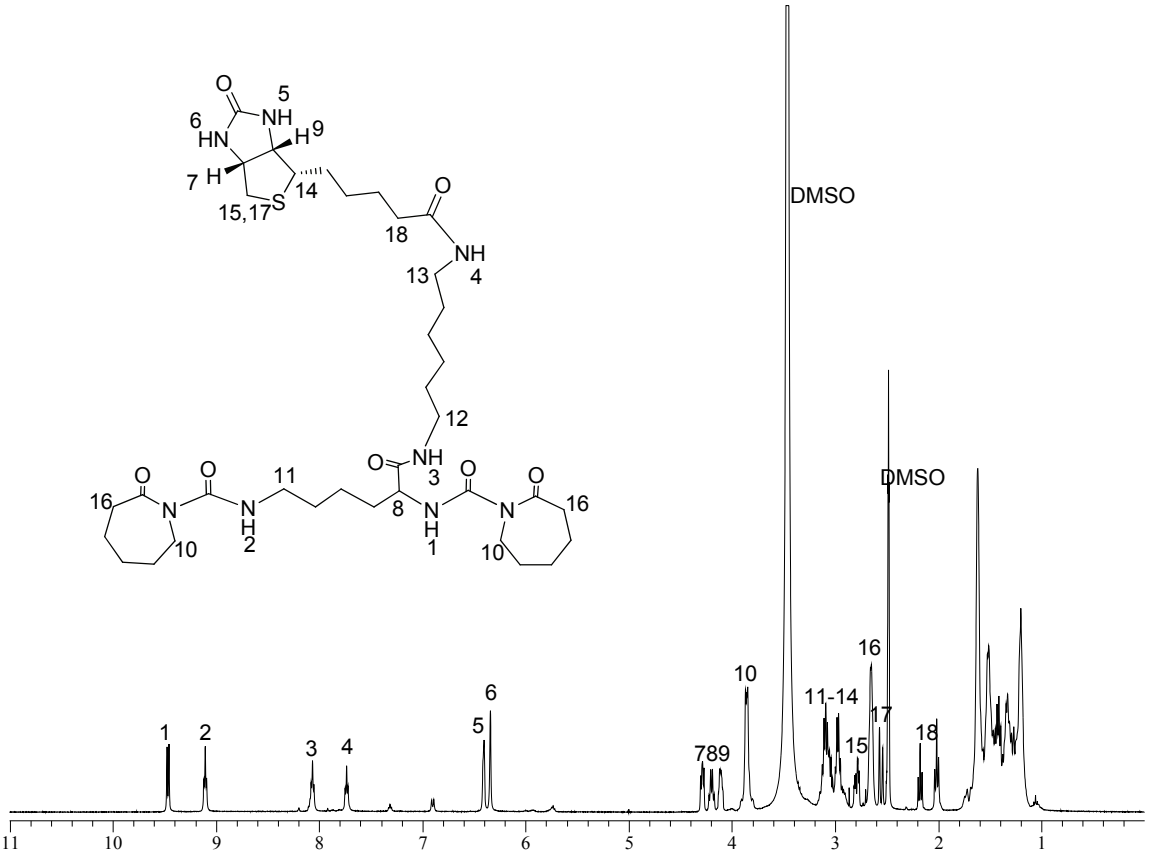


Figure 6.1 ^{1}H-NMR spectrum of BLDI-C_6-biotin

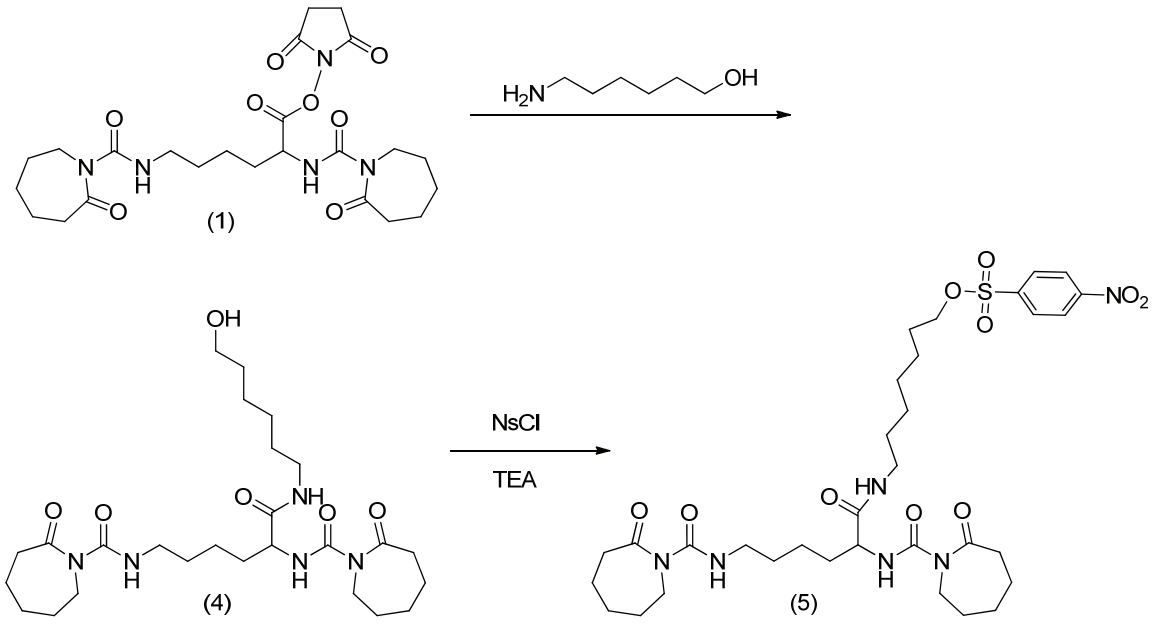
6.3.2. Synthesis BLDI-NH-PMOX-biotin

With a C_6 spacer between a polymer backbone and a bio active compound the accessibility may be insufficient. Therefore, longer spacers between the bio group and backbone may be crucial for binding or function performances.[13,14] Similarly as required for biomaterials, the spacer should be nontoxic. Water soluble spacers are desired to improve the accessibility of the bio-active groups in the aqueous environment of the body. There are two kinds of polymers, poly(ethylene glycol) (PEG) and poly(2-alkyl-1,3-oxazolins) (POX), that meet these requirements. PEG, prepared by means of anionic

polymerization, is one of the best investigated biomedical polymers. In this study we chose the other polymer poly(oxazoline) as our spacer polymer, which has been prepared by a living cationic polymerization leading to narrowly distributed macromolecules.[15] An advantage of polyoxazolines is that various functional groups, such as double bonds, epoxy-groups, siloxanes, hydroxyl groups and amines, can conveniently be introduced as end-groups.[16] It was established that, for instance, the living cationic polymerization of 2-alkyl-1,3-oxazolines started with bifunctional initiators and terminated with various nucleophiles led to well-defined macromolecules functionalized at both terminal ends.[17]

6.3.2.1. Synthesis of derivatives of BLDI-OMe

Here, we explored the option to couple biotin via a spacer onto polyurethane backbones. The caprolactam blocked lysine diisocyanate methyl ester was used as starting material to prepare an initiator for the polymerization of 2-methyl-1,3-oxazoline (MOX). The living polymer was terminated with hexamethyleneteramid, to introduce an amine end group. The NH_2 end group was needed for coupling biotin-NHS onto the blocked diisocyanate functional PMOX. The synthesis of initiator for PMOX is depicted in scheme 6.4.



Scheme 6.4 Synthesis of BLDI-NH-C_6-OH and of the initiator BLDI-NH-C_6-ONs(5) for the preparation of polyoxazolines

By reacting 6-aminohexanol, with BLDI-NHS a hydroxyl functional blocked isocyanate (BLDI-NH-C_6-OH (4)) was obtained. This intermediate compound was easily nosylated by 4-nitrobenzenesulfonyl chloride (NsCl) at room temperature, forming BLDI-NH-C_6-ONs (5). After isolation and purification steps, the structure of initiator was validated by ^{1}H-NMR in $CDCl_3$. The peaks at 8.39 and 8.19 ppm were assigned to the aromatic protons (Figure 6.2) and the peak at 4.12 ppm was related with CH_2 next the nosylate group. All other signals in the spectrum were in agreement with the expected product. ESI-MS also supported that the desired product was obtained.

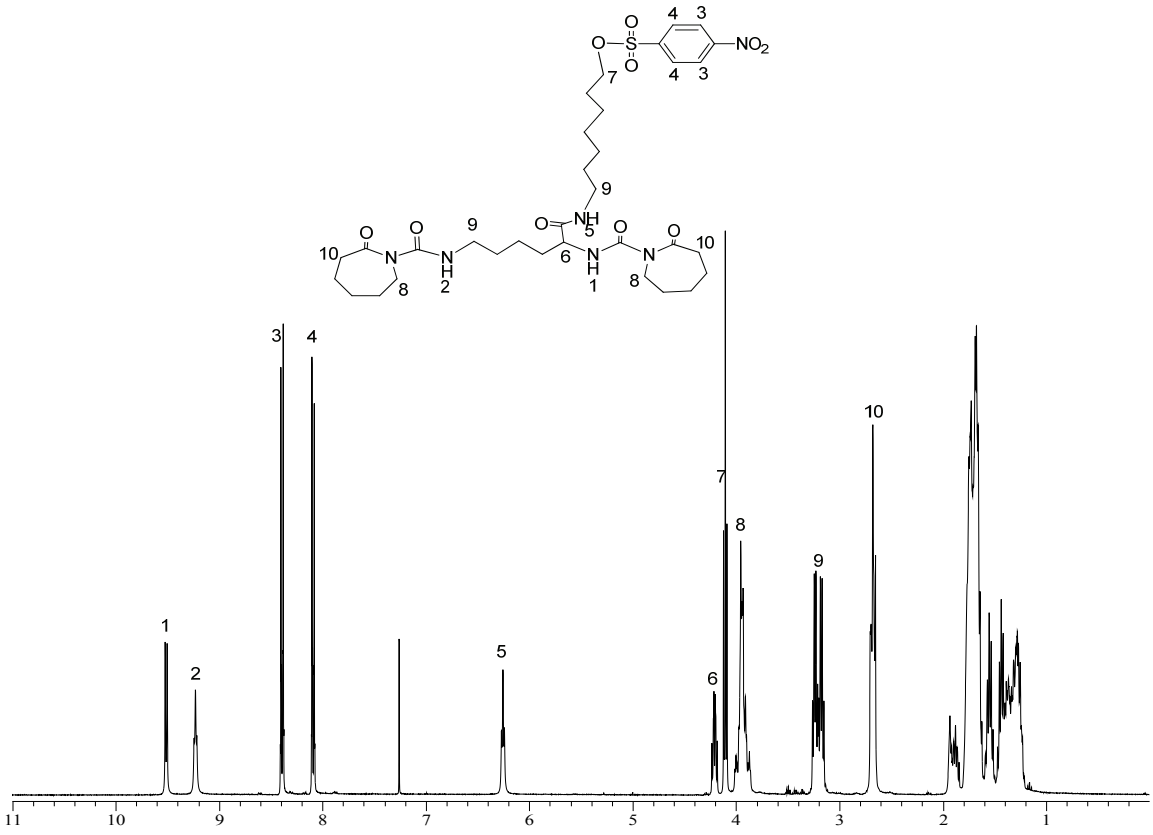


Figure 6.2 ^{1}H-NMR spectrum of initiator (BLDI-NH-C_6-ONs(5)) for preparing polyoxazolines

6.3.2.2. Polyoxazolines comprising a BLDI end group

The initiator was used to polymerize 2-methyl-1,3-oxazoline (MOX) cationically via a ring-opening polymerization mechanism. To study the kinetics, polymerizations were performed in a monomer/BLDI-NH-C_6-ONs ratio of 10.

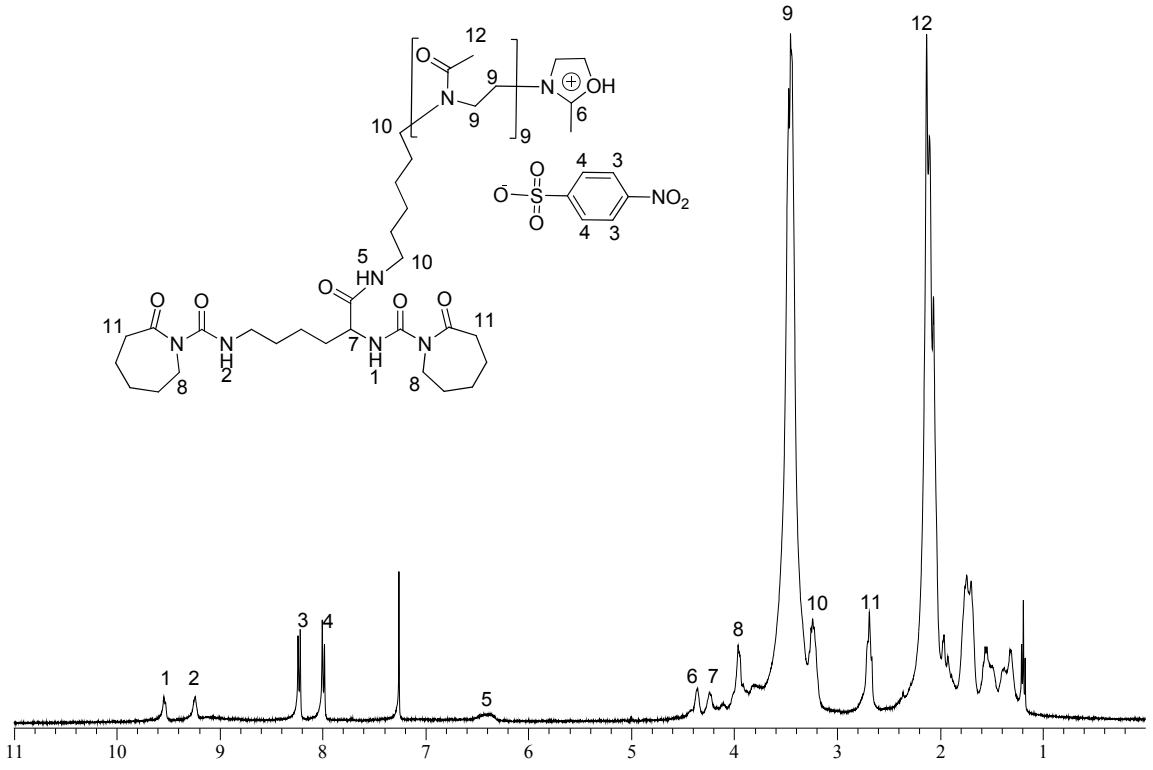


Figure 6.3 ^{1}H-NMR spectrum of the living polymer BLD-INH-PMOX-ONs

Polymerization of 2-oxazoline monomers, using a nosylate initiator, has been shown to have a 100% efficiency.[18] In our case, the blocked lysine diisocyanate nosylate initiator gave indeed a complete initiation during the polymerizations. This was proven by the shifts of aromatic signals of initiator in proton NMR from 8.39 and 8.19 ppm to 8.24 and 8.01 respectively (Figure 6.3). The high intensity peaks at 3.72-3.28 and 2.29-2.00 ppm are related to the repeating unit of PMOX. All other signals in the spectrum were in

agreement with the expected products. This meant that the blocked isocyanate groups survived the polymerization conditions.

Kinetics of 2-methyl-1,3-oxazolines (MOX) polymerizations, using the nosylate initiator, were followed by ^{1}H-NMR measurements (Figure 6.4). Samples of the acetonitrile solutions were taken within selected time intervals during the polymerization and dissolved in $CDCl_3$ for analysis.

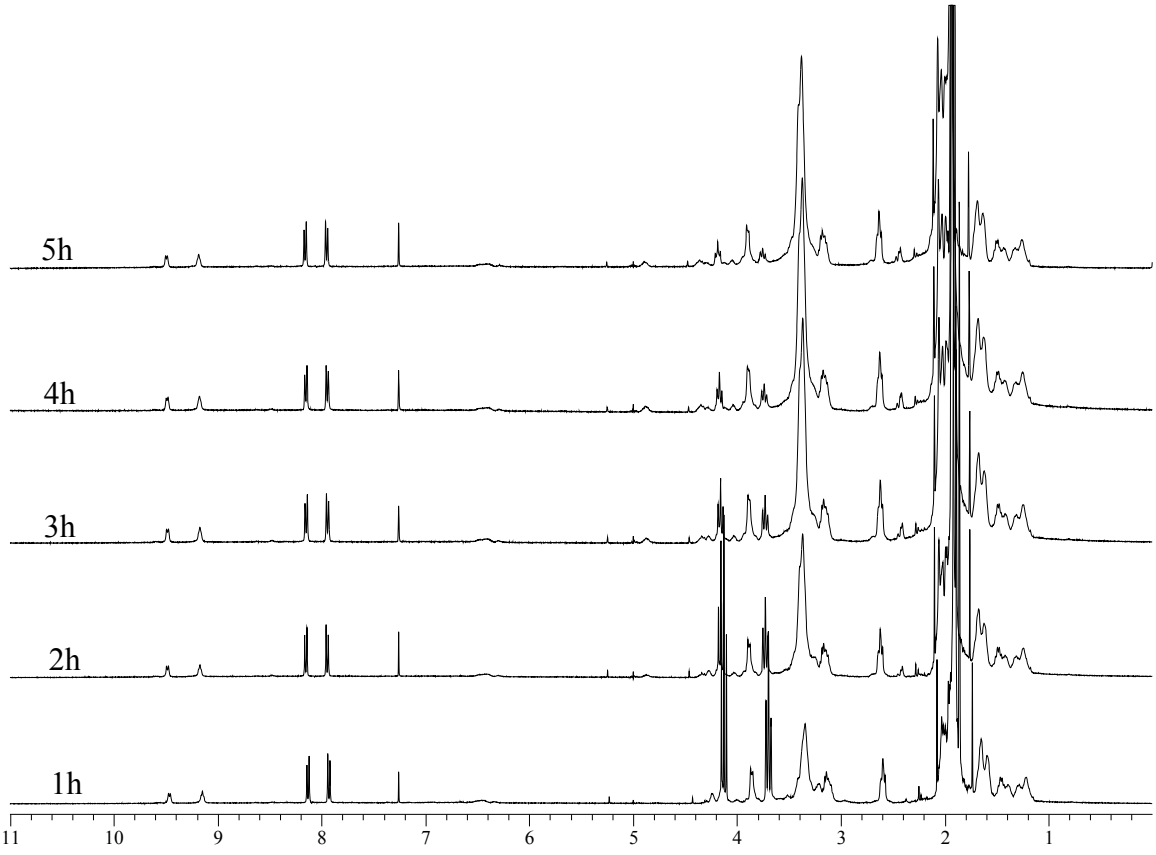


Figure 6.4 ^{1}H-NMR spectra of PMOX samples at various time intervals during the polymerization of MOX, initiated by BLDI-C_6-ONs (Calculated DP=10, concentration 10%w/V, monomer/ solvent).

The big peaks at around 2 ppm were assigned to acetonitrile. The peaks at 9.47, 9.16, 3.86 and 2.59 ppm were preserved during the reaction, and were related to the blocked diisocyanate groups. The peaks centered at 4.13 and 3.69 ppm represented unreacted

MOX, which intensity decreased with increasing of reaction time with respect to the aromatic signals (8.13 and 7.94 ppm). At same time, the intensity of peak centered at 3.34 ppm (repeating unit of PMOX) increased with increasing of time. The ^{1}H-NMR signals were used to calculate the degree of polymerization (DP) (Figure 6.5).

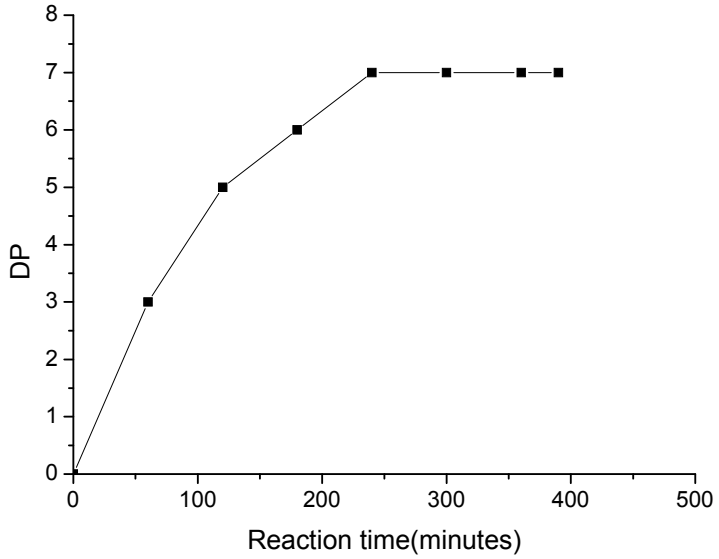


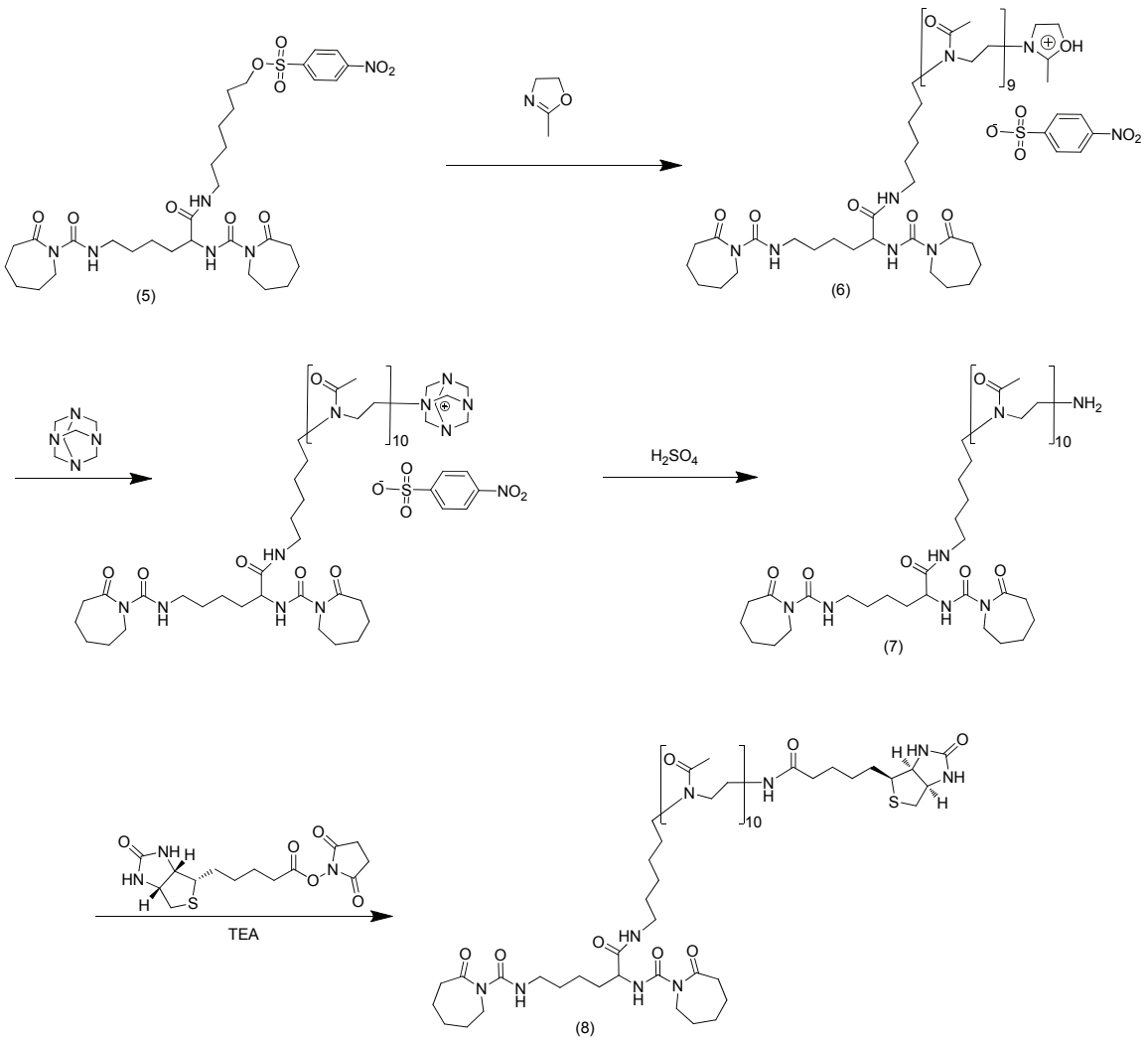
Figure 6.5 Degree of the polymerization of MOX, initiated by BLDI-NH-C_6-ONs (Calculated DP=10, concentration 10%w/V, monomer/ solvent).

This graph shows that the polymerization was quite rapid. It can be seen that after 4h the polymerization rate leveled off and the DP of polymer became constant. The highest DP obtained was 7, which was lower than the calculated value based on the [monomer]/[initiator] ratio of 10. A possible explanation for this discrepancy could be the presence of moisture. Traces of water in a cationic polymerization can give proton initiating, resulting in more polymer chains with lower molecular weights.

The molecular weights and polydispersities of BLDI-NH-PMOX-ONs were determined by using size exclusion chromatography (SEC). The molecular weights were in agreement with the ^{1}H-NMR results, as shown in Table 6.1. The polydispersities as obtained by SEC were around 1.4. This value is quite high for living cationic polymerization. However, this is not unusual for low molecular weight polymers (DP=10)

and if the initiation step is not much faster than the propagations steps. Depending on the applications, various lengths of spacers can be chosen.

Introduction of an amino functional group at the chain end is, according to the literature[19], possible by end-capping the living polymer with ammonia. However, we found that the yield of this reaction was very low. End-capping of the living polymer by hexamethylene tetramine (HMTA), resulted in a convenient method to introduce primary amino functional end group, after acid hydrolysis of the HMTA moiety of the HMTA functional polymers.[20] By subsequently reacting the amino functional PMOX with biotin-NHS, a PMOX-spacer comprising biotinylated blocked lysine diisocyanate was successfully obtained (Scheme 6.5).



Scheme 6.5 Polymerization of MOX with BLDI-functional initiator, termination of the living PMOX with hexamethylene tetramine, followed by hydrolysis to obtain BLDI-C_6-NH-PMOX-NH_2 and the modification thereof with biotin.

The successful end-capping of the living polymers with HMTA and the subsequently formations of polymers with NH_2 end group were first demonstrated by the ninhydrin staining method on the TLC plate (Figure 6.6).[11] Ninhydrin gives selectively a discoloration in the presence of primary amines. The appearance of a violet color on the TLC plate showed the presence of primary amine group.

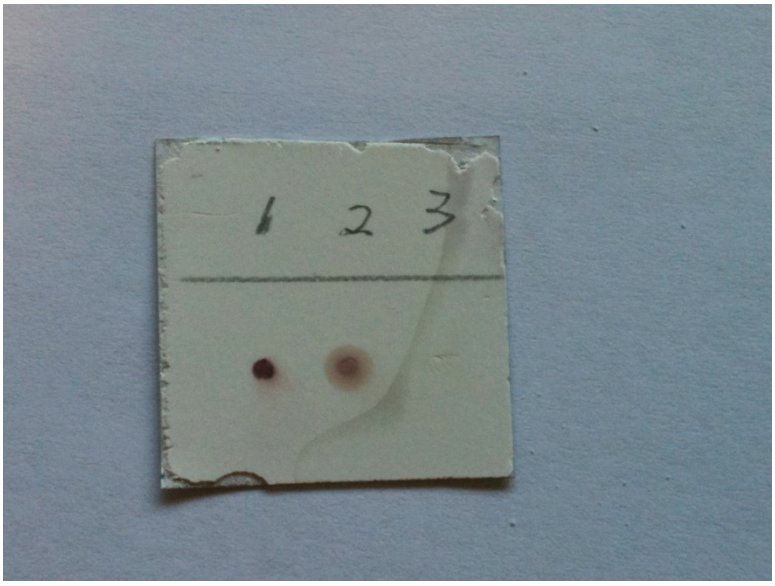


Figure 6.6 Detection of primary amines on TLC slide with ninhydrin: 1=hexylamine; 2=BLDI-PMOX-NH_2; 3=BLDI-PMOX-ONs.

The efficiency of the termination reaction with HMTA and the subsequent biotin coupling reactions were determined by ^{1}H-NMR. Figure 6.7 displays the ^{1}H-NMR spectra of BLDI-NH-PMOX-NH_2 and BLDI-NH-PMOX-biotin. The absence of aromatic signals (8.13 and 7.94 ppm) is a further evidence of the successful termination reaction in high yields with HMTA. This can be seen by comparing Figure 6.7 (a) with Figure 6.3. The peaks at 6.40 and 6.34 ppm belong to the NH from biotin ring which is very characteristic in ^{1}H-NMR spectrum of biotin, as discussed previously.

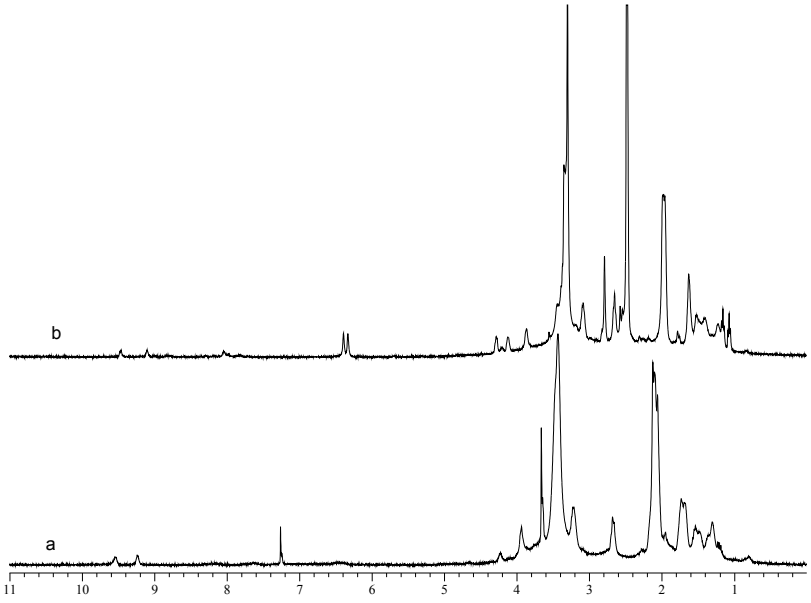


Figure 6.7 ^{1}H-NMR spectra of a: BLDI-NH-PMOX-NH_2 in $CDCl_3$ and b: BLDI-PMOX-biotin in DMSO-d_6.

All the other signals in the spectra were as well in agreement with the expected products. Although strong bases and acids were used during the reaction, no hydrolysis of the PMOX spacer happened. This was further supported by SEC measurement as shown in Table 6.1. The molecular weights of the polymers did not change significantly during the modification reaction. Importantly, the blocked diisocyanate groups were still available to make new biotinylated polyurethanes.

Table 6.1 Molecular weight of BLDI-NH-PMOX

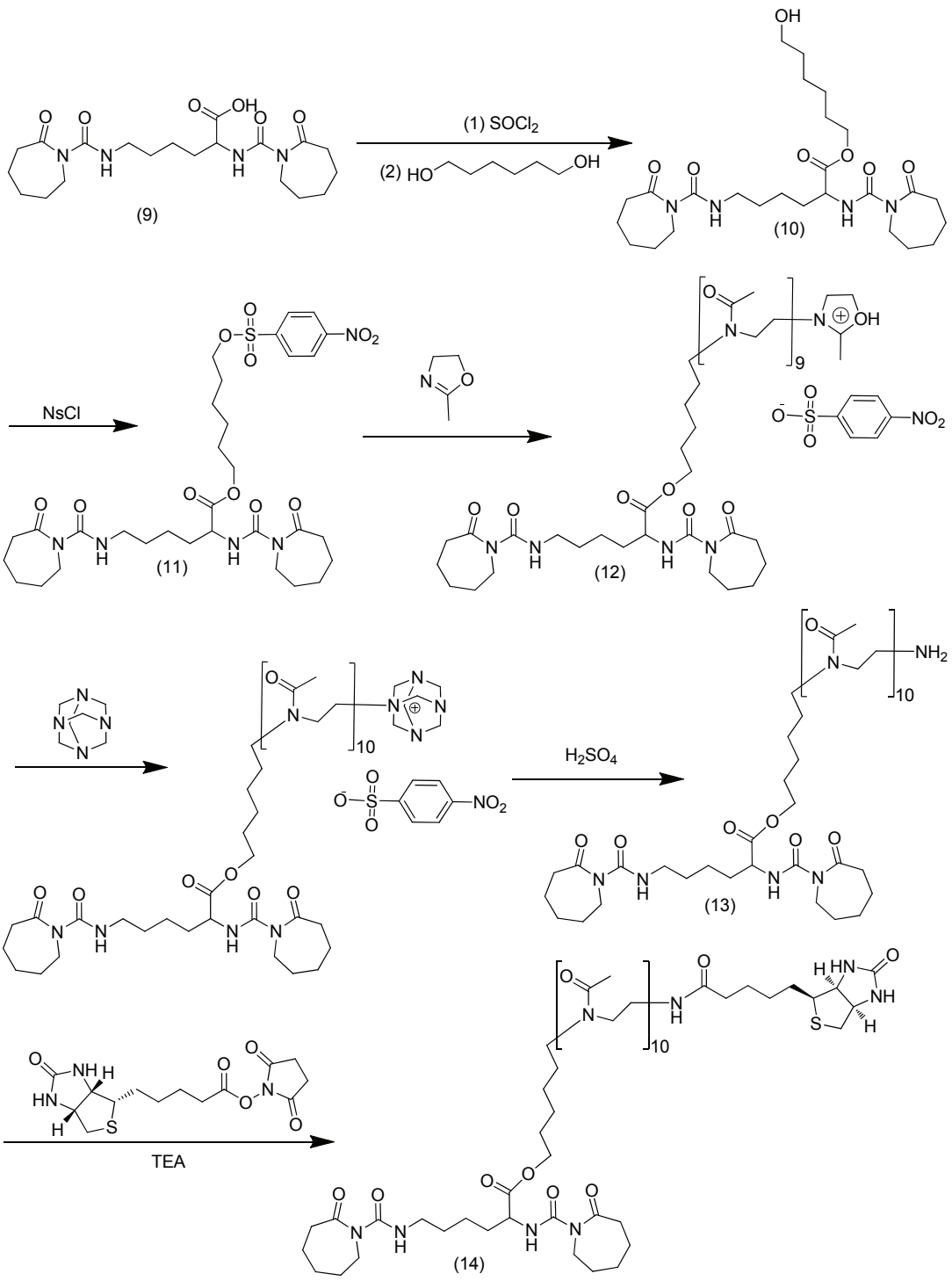
Sample	DP calculated	NMR		GPC		
		DP	M_n(Da)	DP	M_n(Da)	PDI
BLDI-NH-PMOX-ONs	10	9	1,501	11	1,658	1.4
BLDI-NH-PMOX-NH_2	10	10	1,390	13	1,639	1.3
BLDI-NH-PMOX-biotin	10	10	1,615	13	1,796	1.3

During the purification step in this series of experiments (precipitate in diethyl ether), low molecular weight products were removed. This could explain why the DP of PMOX in

SEC measurements in this series was higher (DP = 10) than in the aforementioned kinetic study (DP = 7). The molecular weights obtained from ^{1}H-NMR measurements are in the same range as those obtained by SEC.

6.3.3. Synthesis BLDI-O-PMOX-biotin

In BLDI-**NH**-PMOX-biotin we selected an amide linkage between the spacer and the caprolactam blocked lysine diisocyanate, because of the high stability of amides. But the electron withdraw ability of amide group, which is located close to one of blocked diisocyanate groups (Scheme 6.3 and 6.4), might influence the reactivity of that blocked isocyanate with the hydroxyl groups of polyols during the polyurethane synthesis.
When a side group was coupled via an ester linkage, we showed earlier (chapter 3) that the hydroxyl groups of PCL reacted well with the blocked isocyanate groups. By this route poly(ester-urethanes) were successfully synthesized from caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe) and polycaprolactone530. In order to get decent polymerization rates, the PMOX-spacer was coupled onto the blocked lysine diisocyanate via an ester linkage. In Scheme 6.6, the preparation of a blocked lysine diisocyanate functional initiator with such an ester linkage, the polymerization of MOX, the termination with hexamethylene tetramine, the hydrolysis of the HMTA and the attachment of biotin are depicted.



Scheme 6.6 Reaction scheme of the preparation of blocked lysine diisocyanate functional initiator, the polymerization of MOX, the termination with hexamethylene tetramine, the hydrolysis of the HMTA and the attachment of biotin.

By reacting an excess of 1,6-hexanediol with BLDI-OH (9) a hydroxyl functional blocked isocyanate (BLDI-O-C_6-OH (10)) was obtained. Similarly as with BLDI-NH-C_6-OH (4), this intermediate compound was easily nosylated by 4-nitrobenzenesulfonyl chloride (NsCl) at room temperature, forming BLDI-O-C_6-ONs (11). As with BLDI-NH-C_6-ONs, BLDI-O-C_6-ONs also gave complete initiation during the polymerizations. The same procedures were used, as with the amide-linkage comprising initiator, to introduce amino, as well as biotin end groups. The ^{1}H-NMR results are summarized in Figure 6.8, in which the spectra are given of BLDI-O-C_6-ONs, BLDI-O-PMOX-ONs, BLDI-O-PMOX-NH_2 and BLDI-O-PMOX-biotin. Except for the presence of an ester linkage, these polymers were the same as with the amide comprising polymers, which we described earlier in this chapter. All the signals in the spectra were in agreement with the expected products.

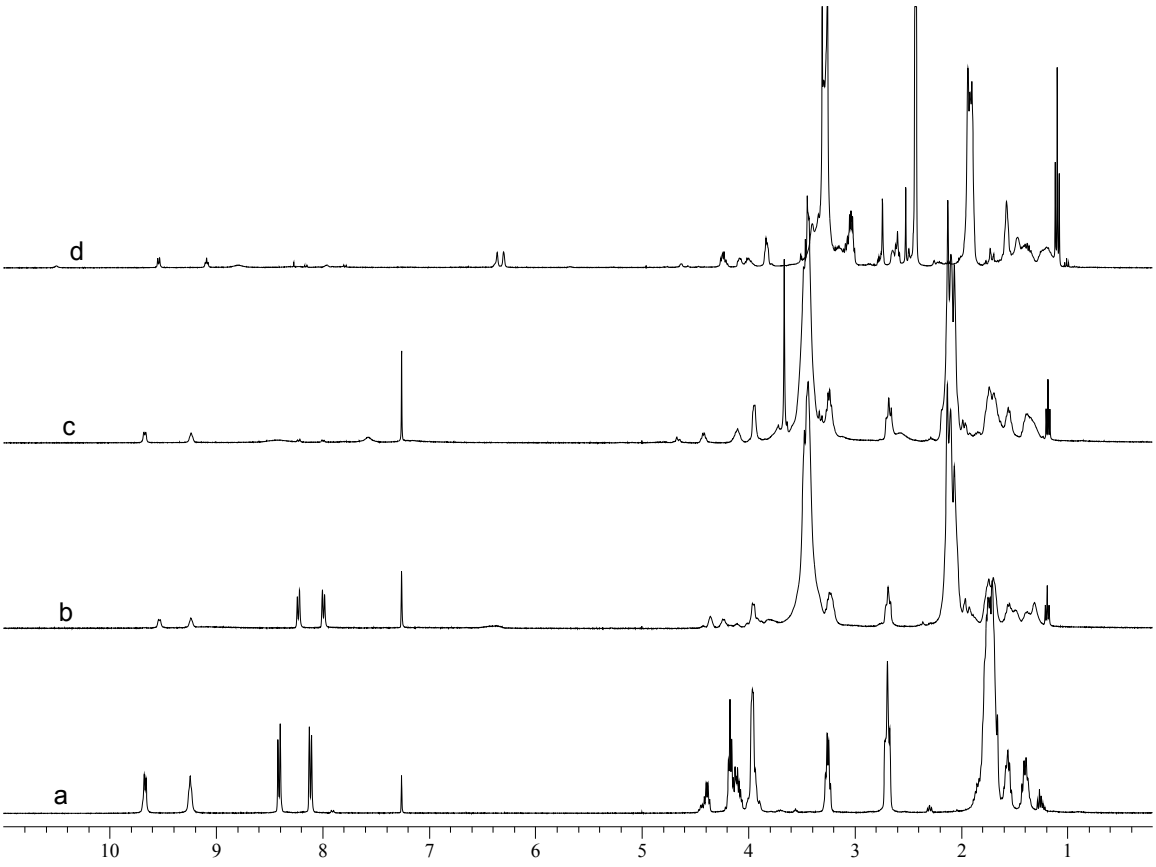


Figure 6.8 ^{1}H-NMR spectra of a: BLDI-O-C_6-ONs; b: BLDI-O-PMOX-ONs; c: BLDI-O-PMOX-NH_2; d: BLDI-O-PMOX-biotin.

In summary, the compounds BLDI-NH-C_6-biotin, BLDI-NH-PMOX-biotin and BLDI-O-PMOX-biotin were successfully synthesized, while the blocked diisocyanate groups were still available to make new biotinylated polyurethanes.

6.3.4. Synthesis of biotinylated polyurethanes

Our homemade caprolactam blocked lysine diisocyanate methyl ester reacted with polydiols to form polyurethanes, as has been described in former chapters. In an attempt to synthesize biotinylated polyurethanes, the biotinylated blocked lysine diisocyanate BLDI-NH-C_6-biotin, BLDI-NH-PMOX-biotin and BLDI-O-PMOX-biotin were heated at 160 ^{o}C for 24h in bulk with PCL530, as a representative polyester diol. The polymerization, as evidenced by an increase of the viscosity, proceeded in a similar way as with the non-functional blocked lysine diisocyanate. The corresponding polymers were characterized by NMR (Figure 6.9) and SEC (table 6.2) analysis. In the ^{1}H-NMR spectra, the peaks at 6.40 and 6.34 ppm (pink square) were observed in all biotinylated polymers. These peaks belong to the NH from biotin ring which are very characteristic in the ^{1}H-NMR spectrum of biotin comprising compounds. It means after polymerization biotin was still there. This was expected considering the stability of the amide linkage between biotin and PMOX, and because of our experience that the methyl ester group of the blocked lysine diisocyanate survived in the polymerization with PCL. The peak centered at 2.25 ppm, originating from PCL530, was also present in spectra of the polyurethanes, but was not present in the spectra of the biotinylated monomers/oligomers.

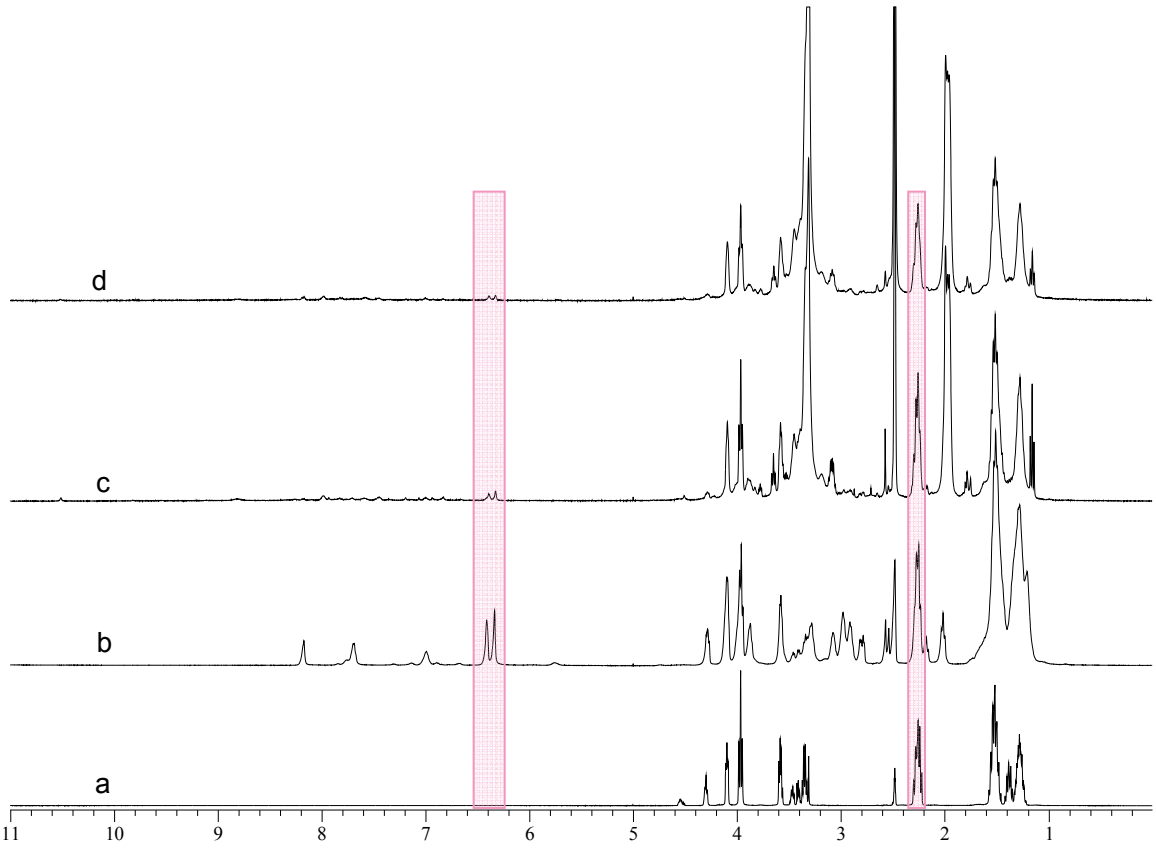


Figure 6.9 ^{1}H-NMR spectra of a: PCL530 in $CDCl_3$ and b: PU-NH-C_6-biotin, c: PU-NH-PMOX-biotin and d: PU-O-PMOX-biotin in DMSO-d_6.

Biotinylated polyurethanes with and without spacer were prepared, but the molecular weights were rather low (table 6.2). However, the final aim of this work was to make crosslinked materials and for that molecular weights of about 3 to 5,000 Da were desired. Moreover, it is not needed that every monomer is provided with biotin groups. Copolymers with non-functional diisocyanates are a possible route to obtain higher molecular weights, if necessary. Here we limited ourselves to demonstrate the concept on how to synthesize biotinylated monomers, followed by a polycondensation to obtain biotinylated polyurethanes. Future work will be focused on specific applications of these novel polymers.

Table 6.2 Molecular weight of PU-C_6-biotin, PU-NH-PMOX-biotin and PU-O-PMOX-biotin

sample	M_n(Da)	M_w(Da)	PDI
PU-C_6-biotin	3,421	8,155	2.4
PU-NH-PMOX-biotin	4,778	13,349	2.8
PU-O-PMOX-biotin	5,684	16, 732	2.9

6.4. Conclusion

The NHS activated ester group of our homemade caprolactam blocked lysine diisocyanates (BLDI) was used to provide BLDI with primary amino or hydroxyl groups. The pendant amino group was used to couple biotin, yielding biotinylated blocked lysine diiocyanate (BLDI-NH-C_6-biotin). Longer spacers between the bio-active group and polyurethane polymer backbone were prepared by nosylation of the hydroxyl pedant group of blocked lysine diisocyanate with 4-nitrobenzenesulfonyl chloride, forming BLDI-NH-C_6-ONs. This compound was used as initiator for the ring opening polymerization of 2-methyl-1,3-oxazolines (MOX). With this highly efficient initiator blocked lysine diisocyanates with living poly(methyl oxazoline) side chains were obtained (BLDI-NH-PMOX). End-capping of the living polymer by hexamethylene tetramine (HMTA), resulted in a convenient method to introduce in a nearly quantitative yield primary amino functional end group. Next, biotin was coupled onto BLDI-NH-PMOX-NH_2 to form BLDI-NH-PMOX-biotin. The amide linkage between the BLDI and the spacer is located next to the blocked isocyanate and had an influence of the reactivity of the neighboring blocked isocyanate group. Therefore, also a biotin functional polymer with ester group between the blocked isocyanate and the polyoxazoline (BLDI-O-PMOX-Biotin) was successfully synthesized.

All three synthesized biotinylated blocked lysine diisocyanates were polymerized with PCL530 to prepare the corresponding polyurethanes. Coupling of biotin groups to our polyurethanes would allow immobilizing biomolecules, like peptides or protein, through biotin-avidin interactions. It gives a huge amount of possibilities to diversify the applications, which need to be studied in the future.

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Summary

Due to a wide range of physical and chemical properties, polyurethanes (PUs) are used in numerous applications like coatings, foams, fibers, engineering plastics and biomaterials. The use of biomaterials for medical implants is rapidly growing due to the aging of the world population and because it is not accepted anymore to live with loss of function due to trauma or wear. There are generally two classes of materials in use. For long lasting applications, like for tubes, the polymers should be stable under physiological conditions. In contrast, for tissue engineering the polymers should be biodegradable in a controlled way. Depending on the composition, polyurethanes can fulfill all these requirements and have to be selected accordingly.

It is convenient to synthesize polyurethanes from diisocyanates (DIs) since the DIs are reactive towards alcohols at low temperature, when catalyzed, and with amines without catalysts. However, there are several major drawbacks. The high reactivity and toxicity of isocyanates do not allow contamination of the body by traces of remnants .

More importantly, due to the high reactivity, it is not possible to have or to introduce moieties with active hydrogens, like hydroxyl groups, amine of acids, while keeping the isocyanate groups intact. Temporarily blocking the isocyanate groups could be an attractive alternative, while still affording the same polyurethanes with polyols. They are less reactive and therefore possibly suitable to introduce bio-active moieties. The only difference is that the polymerization rate is slower due to the protective group. Above a certain threshold temperature hydroxyl groups are able to substitute the blocking group, yielding polyurethanes. The advantage of blocked isocyanates is that they are stable below 100 ^{o}C, allowing chemical modifications, which are impossible with unprotected isocyanates.

This thesis studies the synthesis of linear or crosslinked polyurethanes from caprolactam blocked lysine diisocyanate (BLDI), which were provided with functional side groups. A novel synthetic methodology to prepare BLDI was developed. By making use of the expected stability of BLDI and the presence of a pendant ester group, the introduction bio-functional side groups like GRGDG and biotin via that pendant ester group was

studied. Amorphous polymer networks were studied to avoid the presence of remnant polymer crystals after degradation. Some preliminary experiments with human cells were employed to demonstrate the biocompatibility of the resulting polyurethanes.

Chapter 2 present an efficient route for the synthesis of caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe) and the derivatization and a detailed characterization thereof. The synthesis of BLDI-OMe was based on the highly selective reaction between compounds comprising primary amines and carbonyl biscaprolactam (CBC). In a highly selective reaction step only one of the two caprolactam rings of CBC was substituted by the amino groups of lysine, yielding BDLI-OMe. The structure and the purity of BLDI-OMe were determined by ^{1}H-NMR, ^{13}C-NMR, elemental analysis and HPLC. The accordingly determined purity was higher than 99%. A successful model reaction showed that octanol substituted selectively only the caprolactam blocking group, while leaving the methyl ester unaffected, demonstrating the possibility to make linear polyurethanes with pendant ester groups. It was essential to omit the commonly used tin catalyst, as the ester reacted with octanol in the presence of this catalyst. Crucial for the aim of this work was that conditions were found to hydrolyze the ester group of BLDI-OMe without affecting the blocked isocyanate groups. So, depending on the reaction conditions either the blocked isocyanate group or the ester was reactive. The free acid group could be converted into the N-hydroxysuccinimide (NHS) activated ester. The activated ester allowed further modification with various nucleophiles, as was demonstrated by the substitution of the NHS-group with hexyl amine, as a model reaction.

Chapter 3 demonstrates the difference in selectivity of the blocked isocyanate groups and the methyl ester of caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe) in the reaction with polydiols during the polyurethane synthesis. For that poly(ε-caprolactone) (PCL) and poly(ethyleneglycol) (PEG) were selected as representative examples for the two classes of polyurethanes (PUs). Linear poly(ester-urethanes) were successfully synthesized by reacting PCL with BLDI-OMe, in solution as well as in bulk. In bulk the polymerizations were much faster than in solution. Above 180 ^{o}C, in bulk, branching of the polyurethanes happened. As a result, depending on the conditions, either

linear or branched polymers or even polymer networks could be made with the same monomer mixtures.

Polyethylene glycol was selected to study the polymerization performance of polyethers with BLDI-OMe in bulk. In contrast to PCL, linear poly(ether-urethane) could not be obtained in this case. Even at 125^{o}C (deblocking temperature of caprolactam blocked isocyanate group), the reactivity of hydroxyl groups of PEG towards the ester group was already too high. As a result, poly (ether-urethane) networks were formed on heating. This offered a convenient way to make polyurethane networks, which will be discussed in chapter 4.

Chapter 4 describes the synthesis of amorphous polyurethane networks based on polydiols and our homemade caprolactam blocked lysine diisocyanate methyl ester (BLDI-OMe) by using a one-step processing technology. After mixing the monomers, the curing was performed by heating the samples for 48 hours at 160^{o}C under vacuum. PEG600, PEG1000, PCL530 and PCL1250 were used as polydiols to study the synthesis of non-crystalline PU networks. Only the PU network films based on PEG600 and PCL530 gave non-crystalline materials, as evidenced by their transparency and by DSC measurement. The polymer films swelled but did not dissolved in chloroform, demonstrating the crosslinked topology.

The PU-PEG600 network was more hydrophilic than PU-PCL530 network, which resulted in a higher absorption of water. *In vitro* degradation experiments showed that the PU-PEG600 network degraded completely in 35 days, whereas PU-PCL530 lost about 3.5% of weight during this period. Mechanical measurements revealed that both polymers displayed rubber-like behavior. The lack of yield points in stress-stain curves demonstrated again the amorphous character. The PU-PEG600 and PU-PCL530 networks in this study are potentially suited for scaffolds for soft tissue.

Chapter 5 describes the synthesis of poly (ester-urethanes), comprising activated N-hydroxysuccinimide (NHS) pendant esters, which were prepared from the NHS-activated ester of caprolactam blocked lysine diisocyanate (Chapter 2) and polycaprolactone. The NHS-activated ester offered an enabled technology to prepare bio-active polyurethanes. First, hexyl amine was used, as model compound, to substitute the NHS-group, demonstrating the ability of the concept. Along the same line, an amino-functional, cell-

adhesion promoting peptide, GRGDG, was coupled onto polyurethane backbones. NMR, IR and elemental analysis proved the successful coupling of both, hexyl amine and GRGDG peptide. XPS and water contact angle measurement of spin-coated glass slides confirmed the presence of the grafted peptides, showing a strong change of the surface property of polymer. The GRGDG peptides promoted adhesion of HDF cells, even in the serum-free circumstance. Since these polyurethanes were applied by a (spin) coating technique, they may be used as functional coatings on the implantable devices to promote cell adhesion. PU-NHS can offer a platform to prepare a library of functional polyurethanes.

Chapter 6 explores the possibility for introducing a biotin moiety onto our homemade blocked lysine diisocyanate, with and without a nontoxic long spacer, and to synthesize corresponding polyurethanes. The presence of biotin on polymers would allow immobilization of various biomolecules, like peptides or protein, through biotin-avidin interactions. In a first step the pendant ester group of blocked lysine diisocyanate was converted into a primary amino or hydroxyl group. The pendant amino group was used to couple biotin and, as a result, biotinylated blocked lysine diisocyanate (BLDI-C_6-biotin) was obtained. The pendant hydroxyl group was nosylated by 4-nitrobenzenesulfonyl chloride, forming BLDI-C_6-ONs. This compound was used as an initiator for the ring opening polymerization of oxazolines. A primary amino group at the end of BLDI-C_6-PMOX was obtained by end-capping the living polymer with hexamethylene tetramine. Next, biotin was coupled onto the BLDI-PMOX, to form a BLDI-PMOX-biotin. The polymerization of the biotinylated blocked lysine diisocyantes with PCL530 yielded the corresponding polyurethanes.

Samenvatting

Dankzij het brede pallet aan chemische en fysische eigenschappen worden polyurthanen (PUs) veelvuldig toegepast in coatings, schuimen, vezels, engineering plastics en biomedische materialen. Het gebruik in biomedische materialen neemt sterk toe vanwege de vergrijzing van de bevolking en omdat men het tegenwoordig niet meer accepteert om te leven met een gebrek vanwege slijtage of een ongeluk.

Er zijn twee klassen van biomedische materialen in gebruik. Een voor langdurig gebruik, zoals in slangetjes, die stabiel blijven onder fysiologische condities. Voor tissue engineering daarentegen, is een gecontroleerde degradatie vereist. Polyurethanen kunnen, afhankelijk van hun samenstelling, aan al deze eisen voldoen.

Polyurethanen zijn eenvoudig te bereiden uit diisocyanaten (DIs) met polyolen of polyamines, met en zonder katalysator respectievelijk. Er zijn echter voor biomedische toepassingen nog een aantal beperkingen aan de huidige PUs. Vanwege hun toxiciteit mogen er geen restanten van de diisocyanaten meer aanwezig zijn in het polymeer. Nog belangrijker is echter dat, vanwege de hoge reactiviteit, het niet mogelijk is om functionele groepen te introduceren met actieve waterstof atomen, zoals hydroxyl-, amino- of zuurgroepen, met behoud van de isocyanaatgroepen. Het tijdelijk blokkeren van de isocyanaatgroepen zou voor al die problemen een aantrekkelijke oplossing kunnen bieden, terwijl toch dezelfde PUs gemaakt zouden kunnen worden. De geblokte isocyanaten zouden dan eerst gemodificeerd kunnen worden met bioactieve groepen, om ze dan te gebruiken voor de synthese van PUs, zij het met een wat lagere reactiesnelheid. Onder 100 ^{o}C zijn geblokte isocyanaten stabiel en zouden dan gemodificeerd kunnen worden en boven de 100 ^{o}C kunnen dan met deze gemodificeerde geblokte isocyanaten weer polyurethanen gemaakt worden.

Dit proefschrift beschrijft de synthese van lineaire en gecrosslinkte polyurethanen, op basis van caprolactam geblokt lysine diisocyanaat (BLDI), die voorzien werden van bio-functionele zijgroepen. Hiervoor is een nieuwe synthetische methode ontwikkeld om BLDI te maken. Door gebruik te maken van de hogere stabiliteit van de geblokte isocyanaten en van de aanwezigheid van de ester zijgroep is onderzocht of bio-

functionele groepen, zoals GRGDG en biotin, in PUs ingebouwd konden worden. Er is gekozen om amorfe polyurethaan netwerken te maken om te voorkomen dat er tijdens en na de degradatie van het implantaat kristallijne restanten achter blijven. Om de bio-compatibiliteit van deze materialen vast te stellen zijn experimenten verricht met humane cellen.

Hoofdstuk 2 beschrijft een efficiënte route om caprolactam geblokt lysine diisocyanate methyl ester (BLDI-OMe) te maken en om deze te derivatiseren. De synthese van BLDI-OMe was gebaseerd op de zeer selectieve reactie van primaire amines met carbonyl biscaprolactam (CBC). In deze selectieve reactie werd door de amino groepen van lysine een van de twee caprolactamringen van CBC gesubstitueerd, waarbij BLDI-OMe in hoge opbrengst gevormd werd. De structuur en zuiverheid (> 99%) werden bepaald met ^{1}H-NMR, ^{13}C-NMR, element analyses en HPLC. De succesvolle reacties van octanol met BLDI-OMe, waarbij caprolactam selectief gesubstitueerd kon worden zonder dat de ester reageerde, toonde aan dat de bereiding van lineaire polyurethanen met een ester zijgroep mogelijk zou moeten zijn. Het was daarbij essentieel om geen katalysator te gebruiken, omdat met een katalysator de ester reactief werd. Cruciaal voor het onderzoek in dit proefschrift was dat we condities gevonden hebben om de ester van BLDI-OMe te hydrolyseren zonder de geblokt isocyanaatgroepen aan te tasten. Dus, afhankelijk van de condities konden we of de geblokte isocyanaat of de ester groep laten reageren. De zuurgroep kon vervolgens omgezet worden in een N-hydroxysuccinimide geactiveerde ester. De geactiveerde ester bood de mogelijkheid om diverse nucleofielen te reageren, zoals aangetoond werd met de model stof hexylamine.

In **hoofdstuk 3** wordt de verschil in reactiviteit van de caprolactam geblokte isocyanaatgroep en de estergroep aangetoond door polyurethanen te maken met polyols. Hiervoor werden polycaprolactone (PCL) en poly(ethyleen glycol) (PEG) geselecteerd als representanten van twee klassen van PUs. Lineaire poly(ester-urethanen) werden succesvol bereid van PCL en BLDI-OMe, zowel in oplossing als in bulk. Pas boven 180 ^{o}C trad er in bulk vertakking op. Dus, afhankelijk van de omstandigheden kunnen lineaire of vertakte of zelfs gecrosslinkte polyurethanen gemaakt worden met hetzelfde monomeren mengsel.

Met poly(ethyleen glycol) konden, in tegenstelling tot PCL, geen lineaire polyurethanen gemaakt worden. Zelfs bij 125 ^{o}C, de deblokkeertemperatuur van geblokte isocyanaten, was de reactiviteit van de PEG-hydroxyl groepen met de estergroep al te hoog om lineaire PUs te verkrijgen. Dit bood echter een goede mogelijkheid om meteen gecrosslinkte PUs te maken, hetgeen de doelstelling van dit onderzoek was.

Hoofdstuk 4 beschrijft de synthese van amorfe polyurethaannetwerken, gebaseerd op polyolen en BDLI-OMe in een een-staps technologie. Na het mengen van de monomeren werd de crosslinking uitgevoerd gedurende 48h bij 160 ^{o}C onder vacuüm. PEG600, PEG1000, PCL530 en PCL 1250 werden geselecteerd om niet-kristallijne PU-netwerken te maken. Alleen met PEG600 en PCL530 werden amorfe netwerken verkregen, dat aangetoond werd door hun transparantie en bevestigd werd met DSC. Deze polymeren zwollen in chloroform, maar losten niet op, hetgeen de netwerkstructuur aantoonde.

Omdat PU-PEG600 veel hydrofieler was dan PU-PCL530 absorbeerde dit polymeer netwerk ook veel meer water. *In vitro* experimenten toonden aan dat PU-PEG600 in 35 dagen volledig hydrolyseerde, terwijl PU-PCL530 slecht 3.5wt% gewicht verloor in die periode. De mechanische metingen lieten zien dat deze polymeer netwerken een rubber-achting gedrag hadden. Het ontbreken van een yieldpunt in de stress-stain curven demonstreerde eveneens het amorfe gedrag. Deze polyurethaan netwerken uit deze studie zijn in potentie geschikt voor tissue engineering.

Hoofdstuk 5 beschrijft de synthese van poly(ester-urethanen) met N-hydroxysuccinimide geactiveerde ester zijgroepen, die gemaakt werden uitgaande van de N-hydrosuccinimide (NHS) geactiveerd BLDI (hoofdstuk 2) en polycaprolactone. PUs met NHS-geactiveerd esters boden de mogelijk om bioactieve groepen te introduceren. Nadat de inbouw van hexylamine, als model stof, was aangetoond is vervolgens het celadhesie bevorderend peptide GRGDG gekoppeld aan het polymeer. Met NMR, IR en element analyses werd aangetoond dat de koppeling succesvol verlopen was. XPS en contacthoek metingen aan gespincoate glasplaatjes bevestigde de koppeling door de grote verandering van het oppervlak van de coating. De GRGDG peptiden bevorderde99999n de adhesie van HDF cellen, zelfs onder serum-vrije condities. Omdat deze monsters aangebracht werden via een coating techniek, kunnen deze materialen ook gebuikt worden om implantaten te

coaten om cel adhesie te bevorderen. Deze PU-NHS bieden een platform om een bibliotheek van bio-functionele polyurethanen te maken.

In **hoofdstuk 6** beschrijft de koppeling van biotin aan onze caprolactam geblokte lysine diisocyanaat, via een korte of lange niet-toxische spacer, waarmee vervolgens polyurethanen bereid werden. De inbouw van biotin in polyurethanen biedt de mogelijkheid om, via biotin-avidin interacties, een scala aan bio-moleculen, zoals peptiden en eiwitten, in te bouwen. De eerste stap was om de zuurgroep van BLDI-COOH om te zetten in een hydroxyl- of aminofunctionaliteit. De zuurgroep van biotin werd vervolgens aan de amino zijgroep gekoppeld via een amide binding, waarbij BLDI-C_6-biotin gevormd werd. De hydroxyl zijgroep van BLDI-C_6-OH werd met 4-nitrobenzeensulfonylchloride omgezet in het overeenkomstige nosylaat. Dit nosylaat werd gebruikt als initiator voor de ringopeningspolymerisatie van 2-methyl-2-oxazoline. Een aminogroep aan het einde van PMOX werd verkregen door de polymerisatie te termineren met hexamethyleen tetramine. Vervolgens werd biotin gekoppeld aan de amino eindgroep onder de vorming van BDLI-PMOX-biotin. De polymerisatie van de nieuwe geblokte isocyanaten met PCL leverde biotin gefunctionaliseerde polyurethanen op.

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